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Aims and Scope. This journal covers artificial cells, blood substitutes, and immobilization biotechnology. The emphasis of this journal is to allow for interdisciplinary interactions. Therefore, we welcome approaches based on biotechnology, chemical engineering, medicine, surgery, biomedical engineering, basic medical sciences, chemistry and others. The following areas are particularly welcomed.

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BLOOD SUBSTITUTES:
MODIFIED HEMOGLOBIN

Second of 3 Special Issues (Peer Reviewed) from
Vth International Symposium on Blood Substitutes
San Diego, California, USA
March 17-20, 1993

Guest Editor

Robert M. Winslow, M.D.
University of California, San Diego, California, USA

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Volume 22, Number 3, 1994

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CONTENTS

BLOOD SUBSTITUTES: MODIFIED HEMOGLOBIN

I. LECTURES

II. POSTERS

CONTENTS

CONTENTS

CONTENTS

III. LATE PAPERS

I. LECTURES

INCREASED VASCULAR RESISTANCE WITH HEMOGLOBIN-BASED OXYGEN CARRIERS

J.R. Hess, V.W. Macdonald, C.S. Gomez, and V. Coppes

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ABSTRACT

Purpose: To compare the effects of resuscitation with hemoglobin-based oxygen-carriers and conventional resuscitation fluids on hemodynamics, oxygen transport, and oxygen consumption in an animal model of the use of these fluids in the treatment of hemorrhagic shock.

Protocol: Twenty-eight immature swine were surgically prepared, allowed to recover five days, water deprived for 48 hours, hemorrhaged of 25 ml/kg over one hour, resuscitated promptly with 1) Ringer's lactate, 75 ml/kg, 2) 7% albumin in Ringer's acetate, 25 ml/kg, 3) 9% unmodified hemoglobin in Ringer's acetate, 25 ml/kg, or 4) 9% αα-crosslinked hemoglobin in Ringer's acetate, 25 ml/kg, and observed with three hours of hemodynamic and oxygen transport measurements.

Results: Systemic and pulmonary vascular resistance were increased in hemoglobin-treated animals to more than twice the levels seen in crystalloid- or colloid-treated controls. Oxygen consumption and the rate of correction of lactic acidosis were not increased in hemoglobin-treated animals.
Conclusions: Increased vascular resistance limits the oxygen transport benefit of cell-free-hemoglobin-based oxygen carriers. Cell-free-hemoglobin-induced increases in vascular resistance may place animals' hearts on an unfavorable portion of the Frank-Starling curve as well as complicate further medical treatment by reducing the animals' tolerance to increases in blood viscosity.

INTRODUCTION

Cell-free hemoglobin causes vasoconstriction in isolated vascular rings with intact endothelium [1] and in perfused coronary arteries [2]. Except in the virtual absence of red blood cells [3], hemoglobin solutions have produced hypertension in animals or have not supported an increase in cardiac output with blood volume expansion. Half of all the humans administered hemoglobin in published trials demonstrated hypertension [4], and a recent human trial of a modified hemoglobin was stopped when patient volunteers developed symptoms suggestive of acute pulmonary hypertension [5]. All of these findings imply that cell-free hemoglobin can be expected to increase vascular resistance in many clinical situations.

We have studied hemoglobin-based oxygen carriers in swine, modeling the conditions of resuscitation from hemorrhagic shock in a field medical environment. Specifically, we have produced solutions of modified and unmodified hemoglobin of defined function and high purity, and we have used these solutions in an animal model that consistently demonstrates increased vascular resistance after administration of hemoglobin solutions. We have tried

to understand the implications of the observed increases in systemic and pulmonary vascular resistance on the future development of hemoglobin-based oxygen carriers.

MATERIALS AND METHODS

Primary reports of the experiments described here have been published elsewhere [6]. The production, oxygen-binding properties, and formulation of the hemoglobin solutions used in these studies have also been reported separately [7,8]. The immature swine model was developed at our institution and extensive descriptions of the surgical preparation [9], normal physiology [10], and response to dehydration and hemorrhage [11] have been published.

The renal vascular resistance was calculated from measurements of aortic blood pressure and renal blood flow, which were monitored continuously along with pulmonary artery pressure during the four-hour hemorrhage and resuscitation procedure. Examples of changes in blood pressure after the beginning of the administration of hemoglobin were taken directly from the primary strip-chart records. Cardiac output was measured intermittently by thermodilution. Consequently, the calculated values for vascular resistance are also discontinuous.

The viscosity of hemoglobin cross-linked between Lys-99$\alpha\alpha$ residues with bis(3,5-dibromosalicyl)fumarate ($\alpha\alpha$Hb) mixed with whole pig blood was obtained with a cone-plate viscometer in a previous study [3]. Those data are provided here in greater detail.

Data on the intravascular and whole-body persistence of unmodified Hb and $\alpha\alpha$Hb were calculated from exponential curves fitted to the plasma hemoglobin concentrations.

RESULTS

Resuscitation of hemorrhagic shock with hemoglobin solutions raised
mean systemic blood pressure from 62.3±3 (Mean±SEM) to 140±4 Torr (40%
higher than prehemorrhage levels). The increase in mean systemic blood
pressure in the control groups of swine resuscitated with Ringer's lactate and
albumin solutions was only half as great, rising to 101±6 Torr (close to normal
prehemorrhage levels). In the hemoglobin-treated animals, the blood pressure
achieved this rise after only 2 minutes when approximately 10 ml/kg of
hemoglobin solution had been administered (FIGURE 1). The resultant
hypertension persisted for the full three hours of observation following
resuscitation.

Mean pulmonary blood pressure increased from 15±3 to 40±4 Torr in
the groups resuscitated with hemoglobin solutions. The final pressures were 19
Torr lower in the groups resuscitated with Ringer's lactate and albumin
solutions. Pulmonary hypertension developed with occasionally startling
rapidity (FIGURE 2), and the final pressures exhibited greater animal-to-animal
variation than was seen in the systemic pressures.

Cardiac output did not increase significantly after resuscitation with
hemoglobin solution, changing from 0.16±0.02 to 0.20±0.02 l/min/kg.
Resuscitation with conventional crystalloid and colloid solutions doubled the
cardiac output, increasing it from 0.15±0.01 to 0.35±0.02 l/min/kg. Post-
resuscitation cardiac output values remained constant in the albumin and both
hemoglobin groups, but declined to 0.26±0.03 l/min/kg in the Ringer's lactate
group one hour after resuscitation.

Systemic and pulmonary vascular resistances calculated at all time
points after resuscitation were uniformly more than twice as large in the
hemoglobin-treated groups compared with the groups treated with Ringer's
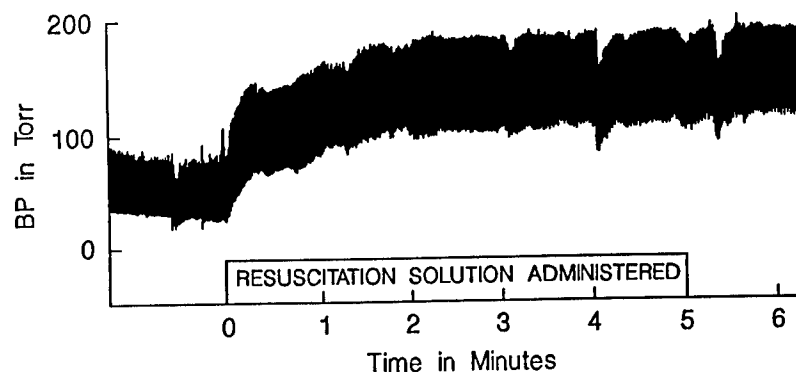
FIGURE 1. Aortic blood pressure during resuscitation with ααHb, 9.9 g/dl, in Ringer's acetate. The tracing was photographed from the original strip-chart recording of the period just prior to, during, and immediately after administration of 25 ml/kg ααHb solution through the jugular vein catheter. Note the very rapid onset of the hypertensive response during the first 30 seconds with the administration of 60 ml of solution containing 0.3 g/kg of ααHb. The full response was attained when only 10 ml/kg had been administered, about 1 g/kg of Hb. Blood pressure rose at an equivalent rate for both uncross-linked HbA_0 and ααHb, lending no support to the argument that the amount of Hb dimer is a determinant of the hypertensive response.
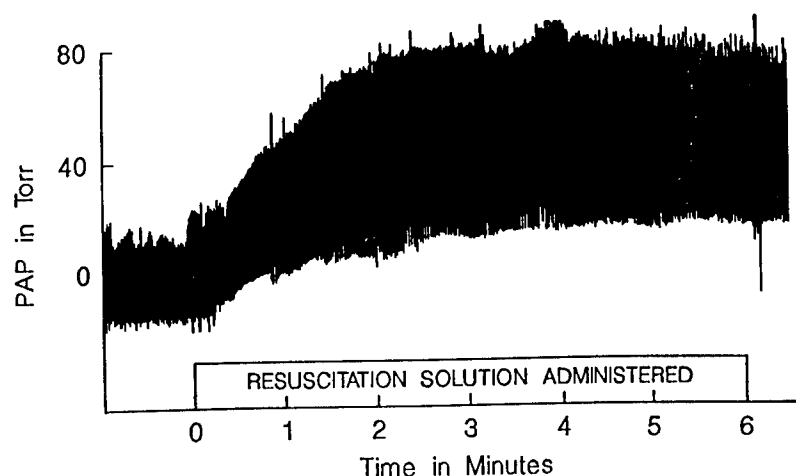
FIGURE 2. Response of pulmonary artery pressure to resuscitation with 9.9 g/dl ααHb in Ringer's acetate. The tracing was photographed from the original strip-chart as in Fig 1, but from a different animal. The animal that died with acute pulmonary edema achieved similar pulmonary artery pressures of 80/30 Torr.

lactate or albumin solution.   Systemic and pulmonary vascular resistance declined approximately 20% after resuscitation with the conventional solutions.

Whole blood viscosity decreased with hemodilution from 3.8 cP at a hematocrit of 30% to 3.2 cP at a hematocrit of 17% with 3 g/dl of ααHb.

Renal vascular resistance was also increased at all time points after hemoglobin administration (FIGURE 3), but was not associated with significant changes in renal function.

The return of 25 ml/kg of blood which had previously been removed at three hours after resuscitation increased intravascular volume and hematocrit. This simultaneous increase in vascular volume and viscosity was well tolerated by all animals.

The initial or redistribution phase rates of change in plasma concentration of unmodified Hb and ααHb were similar with half-concentration times of about 4.5 hours. During the next day, unmodified hemoglobin continued to be eliminated at the same rate, but the whole body half-clearance time of ααHb was 13 hours.

DISCUSSION

"Hemoglobin is a vasoconstrictor," according to Amberson [12], but the clinical implications of this observation have not been explored. Massive hemolysis in humans which occurs with hemolytic transfusion reactions, hemolytic anemias, *Clostridial* sepsis, and falciparial malaria is sufficiently desperate that reliable studies of the vascular resistance changes associated with these conditions are not available. A standard textbook of transfusion medicine says that cell-free hemoglobin is safe, and mentions its development as a blood substitute as proof [13]. However, measurements of vascular resistance in human trials of hemoglobin-based oxygen carriers have never been published.
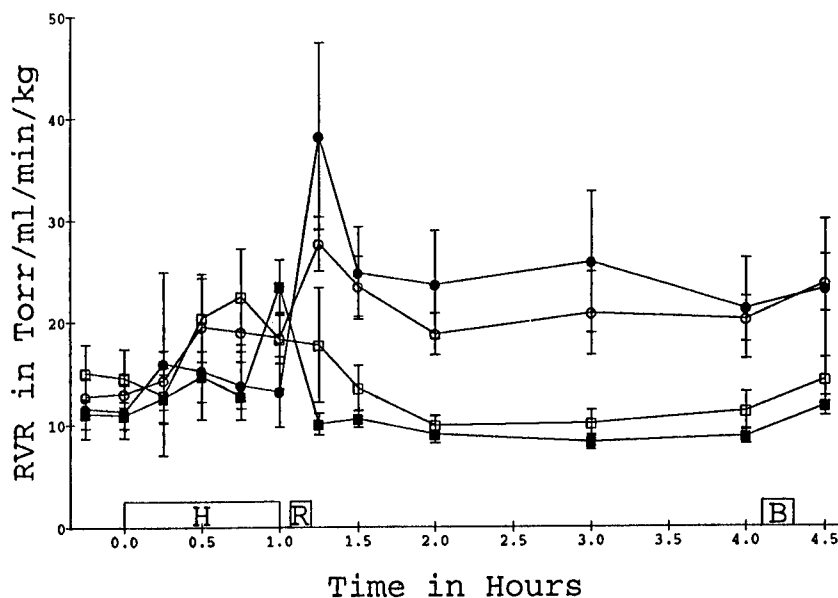
FIGURE 3. Renal vascular resistance (RVR) at discreet time points before, during and just after hemorrhage (H); after resuscitation (R); and after return of shed blood (B). Animals were resuscitated with equivolume amounts of 9.9 g/dl ααHb (●), 9.9 g/dl unmodified hemoglobin (O), or 7 g/dl human serum albumin (■) in Ringer's acetate or with Ringer's lactate at three times the volume of shed blood (□). Values are Mean±SEM.

Previous animal studies of the effects of cell-free hemoglobin have been interpreted to view hypertension, or the failure to increase cardiac output in the face of decreased blood viscosity, as efficacy [14]. The implications of increased vascular resistance on the usefulness of cell-free hemoglobin solutions have thus not been addressed in critical experiments.

We conducted an animal study searching for any toxicity of cell-free hemoglobin that might occur under the anticipated conditions of use in resuscitation. We measured aortic blood pressure, pulmonary artery blood pressure, cardiac output, and renal artery blood flow, because we believed that

these hemodynamic measures might be important in understanding renal tubular and hepatic central-lobular necrosis reported previously [15]. Here, we review the data of that study [6] with specific attention to potential complications of increased vascular resistance.

Swine resuscitated with ααHb exhibited the rapid onset of marked systemic hypertension. The blood pressure rose within seconds of the beginning of hemoglobin infusion and achieved hypertensive pressures after the delivery of less than 0.5 grams of hemoglobin per kilogram of body mass. The blood pressure often achieved its maximum before the completion of hemoglobin infusion and persisted for at least the three hours of hemodynamic observation without diminution. In the one animal that was measured 24 hours later, hypertension persisted.

The hypertension was consistently associated with a doubling of systemic vascular resistance. In the hemoglobin-treated animals, cardiac output changed very little with resuscitation or during the period of hemodynamic observation. Changes in blood pressure may thus be viewed as reflecting changes in vascular resistance. Renal vascular resistance, a component of the systemic vascular resistance, but derived from continuously collected data, was also increased. The addition of more hemoglobin solution after the achievement of the hypertensive plateau did not further raise pressure or reduce renal flow. At the end of the experiment, when the blood which had previously been removed was returned, increases in pressure and reductions in flow were only minimal. The results of these two volume challenges suggest that the effects of hemoglobin on blood pressure and resistance are independent of effects on vascular compliance. Alternatively, the failure to respond to increases in volume raises the possibility that the increased cardiac work places the animal's heart near the apex of the Frank-Starling curve.

In our study, one animal with preexisting ventricular dysfunction died with hypertension and low cardiac output after receiving hemoglobin. In the

absence of methods to treat the increased vascular resistance, human subjects enrolled in clinical trials should be carefully screened for cardiovascular disease.

Pulmonary hypertension also occurred consistently with the administration of hemoglobin solutions. The pulmonary hypertension varied from animal to animal. It achieved its highest pressure during hemoglobin administration then decreased to a lower but still elevated level during the next 30 minutes. The increase in pulmonary artery pressure is dose-dependent only when less than 0.5 g/kg of Hb is administered. This suggests that no protection against pulmonary hypertension would be provided by resuscitation with a clinically significant volume of Hb solution that is less than the amount of blood which has been shed. Pulmonary vascular resistance was also elevated, although the exact degree of elevation is less certain, because resting pulmonary artery wedge pressures were not consistently obtainable in these awake animals.

In our study one animal died with acute pulmonary edema after receiving hemoglobin. The animal had pulmonary artery blood pressure greater than 80/30 Torr. Because the pressures in several other animals were only slightly lower, and because humans in clinical trials of Hb have suffered symptoms suggestive of acute pulmonary hypertension, this reaction needs to be understood and a treatment developed before large scale human trials of hemoglobin are undertaken.

Despite these incidents, the hypertension and increased vascular resistance were usually well tolerated. Hemoglobin administration did not appear to interfere with vascular compliance or limit further treatment with whole blood. The majority of these young, healthy animals thus tolerated the increased vascular resistance, but the reduced cardiac output may have offset the increased oxygen-carrying-capacity of hemoglobin-based resuscitation fluid so that hemoglobin appeared to confer no benefit in this resuscitation model.

The safety and efficacy of cell-free hemoglobin remains in doubt. Over 200 people have been given varying amounts of the solutions with only one reported death, but many individuals have experienced untoward reactions. Even an ideal oxygen-carrying solution may have limited benefits in the resuscitation from acute hemorrhagic shock of young, previously healthy individuals who respond well to volume expansion. Clearly, more research on the basic physiology and toxicity of hemoglobin-based oxygen carriers is needed.

ACKNOWLEDGEMENTS

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REDOX REACTIVITY OF MODIFIED HEMOGLOBINS WITH HYDROGEN PEROXIDE AND NITRIC OXIDE: TOXICOLOGICAL IMPLICATIONS

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28516, USA

The rapid unloading of oxygen to tissue and the prevention of subunit dissociation have been the main concerns in the search for an effective hemoglobin-based red cell substitute. The presence of redox active iron however, raises some questions about its potential to enter into reactions that mediate the formation of cytotoxic oxygen free radicals. We tested the propensity of modified hemoglobins to undergo oxidative damage by peroxide (H_2O_2). We found differences in their susceptibility to oxidative modification and in their ability to form the highly cytotoxic ferryl species. This protein-associated oxidant may be a physiologically important contributor to reperfusion injury. Another potential mechanism of toxicity involves the reaction of cell-free hemoglobin with endothelium derived nitric oxide (NO). Marked hypertensive responses in intact animals infused with some of these hemoglobins were reported. Cell-free hemoglobin has the potential to bind the endothelial generated NO yielding methemoglobin and nitrate, an extremely rapid reaction in vivo. We describe subsequent redox reactions between NO and methemoglobin which may further deplete NO as a biological transducer, leading to greater effects on the extent of endothelial-dependent responses. The consequences of a potential linkage between oxidative toxicity of cell-free hemoglobin and its interaction with NO is addressed.

INTRODUCTION

Oxidation of cell free hemoglobins by superoxide, peroxide and nitric oxide is currently under intense study because of its relevance to cellular damage [1,2]. Superoxide (O_2^-) is released during the spontaneous oxidation of hemoglobin [3]. The formation of hydroxyl radicals ($\cdot$OH) or similar highly oxidizing agents is believed to account for much of the cytotoxic damage in living systems by increased intracellular generation of (O_2^-) and (H_2O_2). This reaction is enhanced by the presence of transition metals such as iron [4].

$$Fe^{II} + H_2O_2 \longrightarrow Fe^{III} + \cdot OH + OH^- \qquad (1)$$
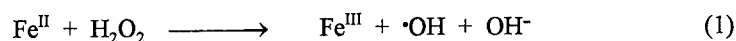
Although hemoglobin has been reported to interact with H_2O_2 in a Fenton reaction like manner (Equation 1) [4], recent studies have demonstrated that H_2O_2 reacts with both oxy and methemoglobin leading to the formation of a higher oxidation state, the ferrylHb (Equation 2 and 3) [5]. In the red blood cell, this highly toxic protein radical is quenched by a comproportionation reaction mechanism (Equation 4) and the resultant metHb is re-reduced by metHb reductase [5].

$$HbFe^{II}O_2 + H_2O_2 \longrightarrow HbFe^{IV}\text{-}OH + OH^- + O_2 \qquad (2)$$
$$HbFe^{III} + H_2O_2 + H^+ \longrightarrow HbFe^{IV}\text{-}OH + H_2O \qquad (3)$$
$$HbFe^{IV} + HbFe^{II}O_2 \longrightarrow 2\,HbFe^{III} + O_2 \qquad (4)$$

The presence of a cell-free hemoglobin in tissues raises the concern of a potential toxicity that may involve its redox active iron [1]. In vivo studies using unmodified hemoglobin have confirmed that the presence of hemoglobin can mediate and/or exacerbate oxidative injury in a variety of tissues [6,7]. Subtle changes in the heme environment, as a result of chemical alteration even at sites distant to heme, can produce appreciable changes in the ability of hemoglobins to react with or generate oxygen free radicals such as superoxide, peroxides and/or protein-radicals [1]. One potential mechanism of toxicity involves the reaction of hemoglobin-based red cell substitutes with oxygen metabolites formed upon reperfusion of ischemic tissues [8]. This could lead to a deleterious pathway in which heme and subsequently toxic iron is released [9].

Changes in the heme environment of some hemoglobins may also determine their reaction kinetics with another free radical, nitric oxide (NO) [2]. This reactivity has special significance because it has recently been recognized that

NO is generated endogenously within a variety of human cell types [10]. The endothelial derived relaxing factor (EDRF), produced within the vascular wall, which signals the endothelium-dependent vascular relaxation, has been identified as NO [10]. Both oxy and deoxy intraerythrocytic hemoglobins ensure the rapid removal of any NO diffusing into the lumen of blood vessels (Equation 5) [11].

$$HbFeO_2^{II} + NO \longrightarrow HbFe^{III} + NO_3^{-} \qquad (5)$$

Cell-free hemoglobin, unlike red blood cells, readily diffuses through the endothelial barriers into the subendothelial space where it has greater potential to bind to the endothelial derived NO, thus limiting the action of NO on the target enzyme, the smooth muscle guanylate cyclase [10]. Cell-free hemoglobins have indeed been shown to alter the vascular tone in vitro and in vivo [12,13]. In the few reported human trials in which a variety of modified hemoglobins were used, hypertension was among the most commonly seen side effects [14].

With the advent of a variety of chemically or genetically altered hemoglobins for in vivo application, their safety and potential toxicity has become of great interest, especially in light of the large amounts necessary for transfusion and the compromised state of the patients to which these products will be given. This paper describes the redox reactions of some modified hemoglobins with H_2O_2 and NO and the implications of such reactions in regard to reperfusion injury and/or vasoconstriction.

MATERIALS AND METHODS

Human Hemoglobin A_0 and two derivatives, one cross-linked between the alpha subunits, DBBF(αXL), and the other, within the beta subunits, DBBF(βXL), were prepared as described [15,16] and were a kind gift from the Letterman Army Institute of Research, San Francisco, CA. Human hemoglobin modified with a pseudo cross-link between the beta subunits, FMDA (PXL), was prepared as described [17] and was a kind gift from Dr. E. Bucci, University of Maryland, Baltimore, MD. Chromatography on DEAE-Sephadex was used to ensure complete removal of other proteins, such as catalase, superoxide dismutase and glutathione peroxidase.

Enzymatic oxidation of hemoglobin by H_2O_2, which was generated at rate of ~120 μM/minute was carried out according to the method of Giulivi and Davies

[5]. In another set of experiments H_2O_2 was added directly to hemoglobins at an increasing molar ratio over heme. In both experiments absorbance changes in the range of 500-700 nm were recorded in a temperature controlled spectrophotometer (Hitachi SU2000) interfaced with a computer for multicomponent analysis [5,18]. The ferrylHb was detected by its derivatization with sodium sulfide (Na_2S) to form sulfhemoglobin [5]. Singular value decomposition (SVD) analysis was carried out to resolve the individual contribution of each hemoglobin component to the visible spectrum according to Shrager [19].

Oxygen equilibrium curves of hemoglobin solutions were determined in the Hemox Analyzer (TCS Medical Products, Southampton, PA) in Hemox buffer (135 mM NaCl, 5 mM KCl and 30 mM TES) and in 0.1 mM phosphate buffer, pH 7.4 at 37°C. Oxygen dissociation rates were determined in an Applied Photophysics SF-17 microvolume stopped-flow apparatus as described [20]. Hemoglobin solutions (30 μM in heme) were deoxygenated by rapidly mixing each hemoglobin with 1.5 mg/ml of sodium dithionite and deoxygenation was followed at 437.5 nm in 0.05 M bis-Tris, pH 7.4 at 20°C. The oxidized (met) forms of hemoglobins were prepared as described [20]. Reaction of ferrihemoglobins (15 μ M heme after mixing) with various concentrations of NO were monitored at 420 nm in an oxygen-free environment using a Durrum-Gibson stopped flow apparatus. Experiments were carried out in 0.05 M bis-Tris, pH 7.0 at 20°C. Rate constants were determined using a non linear least square curve fitting program described by Jonhson et al. [21]. Methemoglobin reduction by NO was carried out in an anoxic spectrophotometric cell. The rates were calculated from the absorbance change at 550 nm (a maximum for the difference between ferric-NO and ferrous-NO).

RESULTS

Hemoglobin-Oxygen Equilibrium and Oxygen Dissociation

The P_{50}s calculated for modified hemoglobins are reported in Table I, and are compared to those for HbA_0. Also reported are the apparent rates (k_{off}) for oxygen dissociation from HbA_0 and modified forms. The increase in the oxygen "off" rates, in the case of DBBF(αXL) and FMDA (PXL) as compared to that of HbA_0, parallels their decreased oxygen affinities. This effect has previously been

TABLE I Oxygenation Characteristics of HbA$_0$ and its Modified Forms

Parameter	HbA$_0$	DBBF (βXL)	DBBF (αXL)	FMDA (PXL)
[a] P$_{50}$(torr); TES	8.0	5.0	25	17
[a] P$_{50}$(torr); PO$_4$	6.0	5.6	27	19
[b] k$_{off}$(s^{-1})	22.8	25	56	36

[a] Partial pressure of oxygen at which hemoglobin is 50% saturated [20].
[b] Apparent first order rate constants for oxygen dissociation [20].

observed with other low affinity hemoglobins which suggest that the major effect on oxygen equilibrium values is brought about by their oxygen "off" rate [1].

Spectrophotometric Analysis of Hemoglobin Reaction with H$_2$O$_2$

Figure 1 shows a comparison of the oxidation profiles of HbA$_0$ and its beta and alpha cross-linked derivatives used in this study with H$_2$O$_2$ produced continuously by the glucose oxidase system as previously described [5]. A decrease in the absorption at 540 and 577 nm is accompanied by appearance of two new peaks at 545 and 580 nm. These spectral shifts have been previously attributed to the formation of the ferrylHb (HbFe^{IV}) [1,5].

Figure 2 illustrates that approximately 2 to 3 minutes after initiation of the oxidation reaction, both HbA$_0$ and the two cross-linked hemoglobins, DBBF(αXL) and DBBF (βXL), established a similar pattern with respect to the early distribution of the oxidation states of the heme iron (i.e. approximately 12-15 % ferrous, 25-30 % ferric, and 55-60 % ferryl). The time scale used in these oxidation experiments does not allow the determination of the sequence of events that occurs within this initial 2-3 minutes. As the reactions progress past this initial oxidation step, clear differences are apparent between the curves for HbA$_0$ and DBBF (βXL), where a significant portion of ferryl (Fe^{IV}) iron becomes reduced to the ferric (Fe^{III}) form and DBBF (αXL) where the distribution of the species stays relatively constant after the initial oxidation phase (Figures 1 and 2).

Enzymatic Oxidation of Hemoglobins
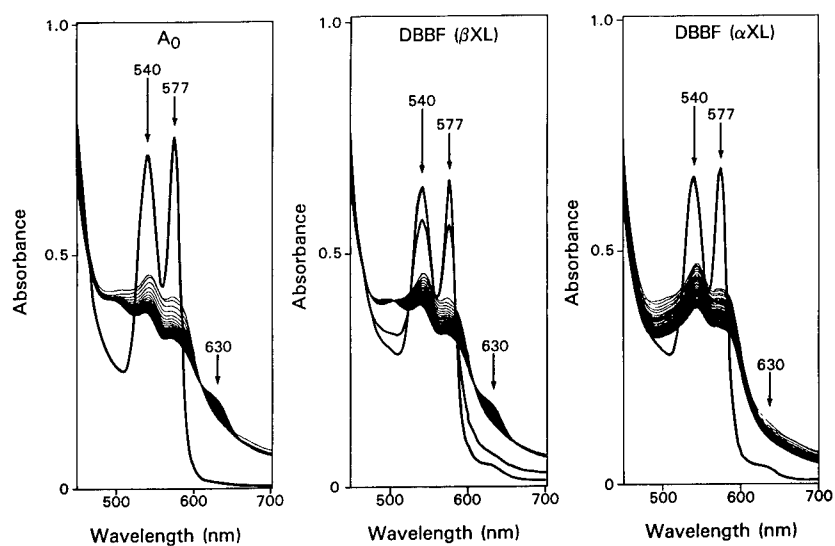
FIGURE 1: Spectral changes during the enzymatic peroxidation of HbA$_0$, DBBF (βXL) and DBBF (αXL). The reaction mixture contained 50 μm (heme) of each hemoglobin in 50 mM phosphate buffer, pH 7.4 and 10 mM glucose. The reactions were carried out at 25°C and were initiated by the addition of 7.5 μg/ml of glucose oxidase. Spectra were recorded at 0 and 1 minute and every 2 minute thereafter up to 61 minutes.
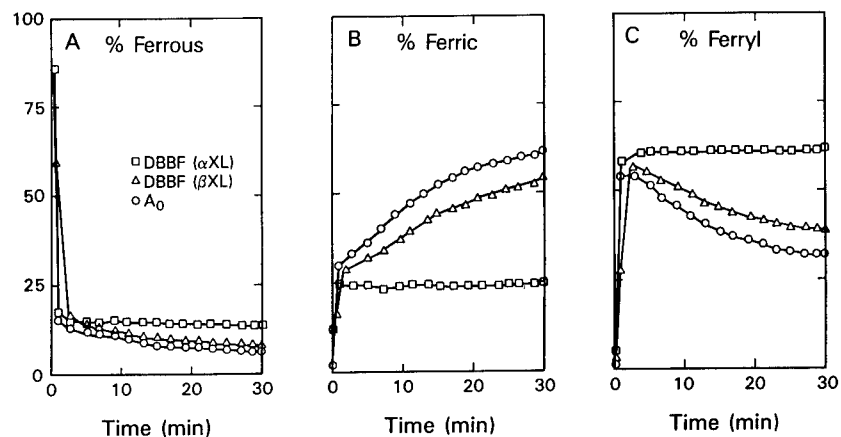
FIGURE 2: Fractional changes in (a) oxy (b) ferric (c) and (c) ferrylhemoglobins during the enzymatic oxidation of HbA$_0$ (○) , DBBF(βXL) (△), DBBF(αXL) (□). Experimental conditions are described in legend for Figure 1. The proportions of these forms were calculated as described in [5].

The formation and presence of ferrylHb can also be demonstrated by derivatization of this species to the sulfhemoglobin form with sodium sulfide as illustrated in Figure 3. In this procedure the hemoglobin, 25 μM (heme), is oxidized with a five fold excess addition of H_2O_2 for five minutes. At this point, excess peroxide is decomposed with catalase (2 μM). Addition of Na_2S to a 2 mM final concentration results in the spectra labeled number 3 in each panel of the figure. This spectrum with its characteristic peak at 620 nm is the result of the reduction of the iron in $HbFe^{IV}$ and incorporation of the sulfur into the porphyrin ring [5]. The spectral changes shown in Figure 3 clearly indicate the formation of sulfhemoglobin in all three hemoglobins with the DBBF (αXL) solution typically containing the highest level.

Another set of oxidations of HbA_0 and DBBF (αXL) was performed where each of the proteins, 20 μM (heme), was oxidized by bolus additions of H_2O_2 in 1:2.5, 1:5, 1:7.5 and 1:10 ratios of heme:peroxide with spectra collected as a function of time. Each of these spectral sequences was subsequently analyzed by SVD (singular value decomposition). Figure 4 shows the results of the reconstruction of the component spectra from these SVD analyses. Each protein exhibits concentration dependent changes in the spectra with varying "ferryl" character of the intermediate spectra (component 2 in the figure). One will note that with respect to component 3 (the final state of the reaction as measured) the HbA_0 curves at all heme:peroxide ratios show a significant reversion of the ferryl intermediate to ferric, as indexed by the peaks at 500 and 630 nm. With DBBF (αXL) only the curve corresponding to the lowest peroxide concentration shows a marked reduction of the ferryl intermediate to the ferric form. These results are in qualitative agreement with the results presented above for the H_2O_2 generation system.

Kinetic Analysis of Hemoglobin Reaction with NO

A typical time course of the initial event that accompanies the mixing of metHb with NO in the stopped flow is biphasic due to the binding of NO to the ferric hemes of the alpha and the beta chains, leading to a NO-ferric heme complex (Equation 6) [2,22].

$$HbFe^{III} + NO \longleftrightarrow HbFe^{III}\text{-}NO \qquad (6)$$
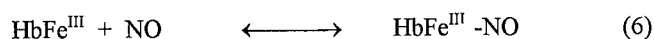
At high concentrations of NO this reaction can be analyzed as a sum of two independent pseudo-first order processes. The apparent rate constants were
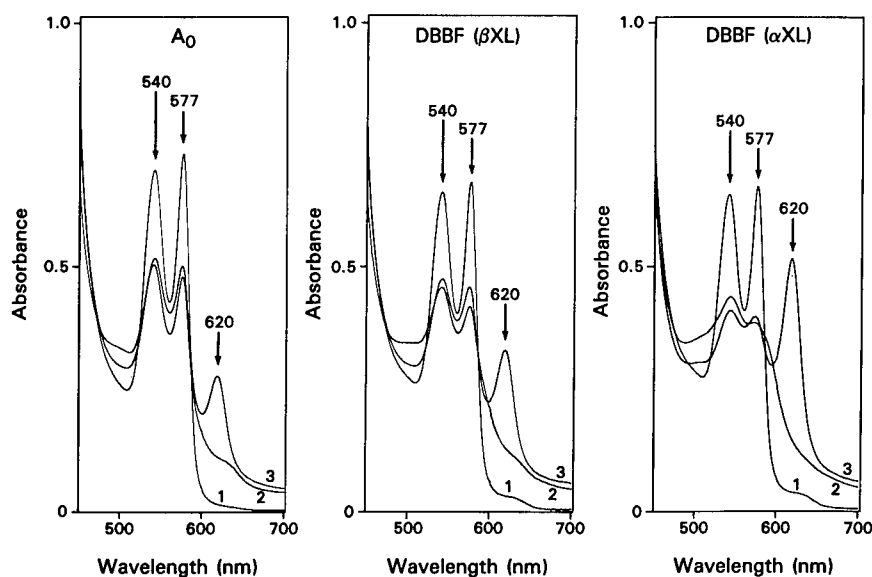
FIGURE 3: Spectral changes during derivatization of ferryl Hb to sulfhemoglobin for HbA_0 (a) DBBF (βXL) (b), and DBBF (αXL) (c). (1) Spectra of oxyHb, 25 μm (heme), at time zero. (2) Spectra of ferryl Hb were obtained after five minutes of oxidation with five-fold molar excess H_2O_2 (250 μm). Catalase (2 μm) was added to decompose excess H_2O_2 (3) Spectra of sulfhemoglobin were obtained after addition of 2 mM Na_2S.

calculated as previously described [20,22] and are reported in Table II. Apparent pseudo-first order kinetic constants (k_1 and k_2) were estimated for the biphasic NO combination curves using a weighted sum of the two exponentials model:

$$A = A_0 \ (n \ \times \ \exp(-k_1 \times \ t) + (1 - n) \ \times \ \exp(-k_2 \times \ t))$$

where n is the proportion of the fast phase, A is the absorbance change at time t and A_0 is the total absorbance change. The kinetic "on" and "off" rates for each fast and slow of these phases were estimated using these apparent pseudo-first order rate constants and the relation which describes the linear change in these values with changing ligand concentration [20,22]:

$$k_{app} = k_{on} \times [NO] + k_{off}$$

RECONSTRUCTED SVD COMPONENT CURVE
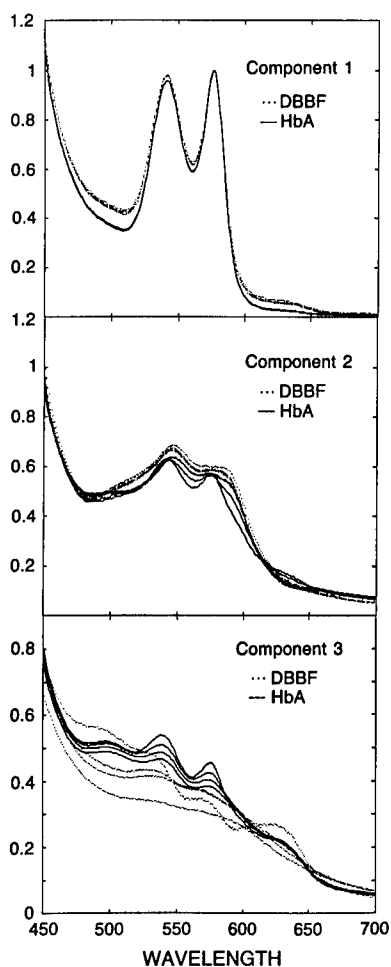
FIGURE 4: Results of the reconstruction of the component spectra from SVD analyses of hydrogen peroxide oxidations of HbA_0 and DBBF (αXL). Each of the proteins, 20 μM (heme), was oxidized by bolus additions of H_2O_2 in 1:2.5, 1:5, 1:7.5 and 1:10 ratios of heme:peroxide with spectra collected as a function of time. SVD analyses were performed and the resulting v matrix transitions (for rank 4) were fitted to the single exponentials. The difference spectra associated with each of the four transitions were computed from the u and s matrices from the original SVD analysis and the matrix of amplitudes (h) produced by the curve fitting procedure [19] by:

$$D = ush$$

Linear combinations of these four difference spectra were used to compute the primary spectra shown above with Component 1 = D1 + D3 + D4, Component 2 = D1 + D2, and Component 3 = D2 where the D's refer to the appropriate difference spectra.

The confidence limits listed are ± 1 standard deviation for each parameter for the data fitted to this equation. As can be seen from Table II, both of these phases in DBBF (αXL) and FMDA (PXL) were altered relative to that of HbA$_0$, suggesting the presence of structural modifications that effect both alpha and beta chains' NO reactivity.

Figure 5 shows the spectral change that follows NO binding to the metHbA$_0$. The initial Fe^{III}-NO spectrum changes to the characteristic spectrum of Fe^{II}-NO shown in this figure. This slow process involves the NO driven reduction of the ferric hemes and the formation of the ferrous-NO complex according to Equation 7 [23].

$$\text{HbFe}^{III}\text{-NO} \longrightarrow \text{HbFe}^{II}\text{-NO} + \text{NO}^+ \qquad (7)$$

The reductive process is essentially first order in Fe^{III}-NO, showing little change if any in the apparent rate constants for a four fold increase in NO concentration. The time courses for this phase for metHbA$_0$ shows simple first order kinetics with a rate constant of $6.3 \times 10^{-4} \, \text{s}^{-1}$. The progress curve for the two modified hemoglobins show biphasic kinetic character and were analyzed as the weighted sum of two independent first-order processes. MetHb-DBBF(αXL) showed rates of $16 \times 10^{-4} \, \text{s}^{-1}$ (84%) and $3.0 \times 10^{-4} \, \text{s}^{-1}$ (16%). Met-FMDA showed rates of $61.4 \times 10^{-4} \, \text{s}^{-1}$ (27%) and $11 \times 10^{-4} \, \text{s}^{-1}$ (73%).

DISCUSSION

Oxidative Toxicity of Cell-Free Hemoglobin

Many biological reactions generate oxygen free radicals that are believed to be cytotoxic [24]. Extracellular hemoglobin may be regarded as a potentially toxic substance, due to its ability to generate oxygen free radicals. Although hemoglobin has been reported to react with H_2O_2 in a Fenton-like manner, recent studies demonstrated the formation of a protein-associated oxidant, the ferryl species [1,5]. This radical has all the attributes necessary to be considered an important species in the free radical damage process [5]. The in vivo generation of ferrylHb and its subsequent removal by the red blood cell reductive system has been demonstrated recently [5]. Reperfusion injury is potentially associated with oxygen free radical generation [24]. In this regard, we have shown in this study that cell-free hemoglobin can indeed form a highly reactive ferryl species in the presence of low H_2O_2 concentration, the ease with which some hemoglobins can

TABLE II Rate Constants for the Combination of NO with metHbA$_0$ and its Modified Forms[a]

Hb(Fe^{+++})	β Chains (fast phase)		α Chains (slow phase)	
	k_{on} (M^{-1}s^{-1})	k_{off} (s^{-1})	k_{on} (M^{-1}s^{-1})	k_{off} (s^{-1})
HBA$_0$	2.10×10^4 $\pm 0.16 \times 10^4$	7.07 ± 1.07	3.67×10^3 $\pm 0.22 \times 10^3$	1.67 ± 0.15
DBBF (αXL)	2.81×10^4 $\pm 0.24 \times 10^4$	10.12 ± 1.49	4.67×10^3 $\pm 0.37 \times 10^3$	2.30 ± 0.23
FMDA(PXL)	3.54×10^4 $\pm 0.28 \times 10^4$	7.26 ± 1.83	6.74×10^3 $\pm 0.30 \times 10^3$	1.65 ± 0.20

[a] Kinetic "on" and "off" rates were calculated as in [22].
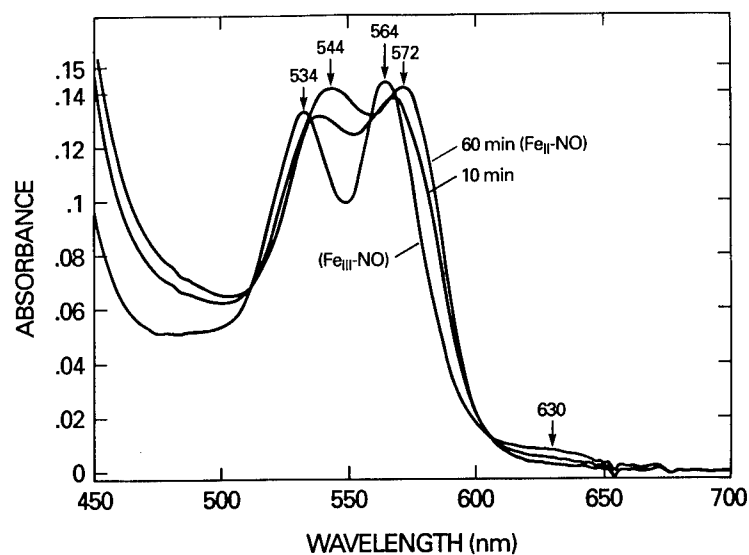
FIGURE 5: Spectral changes during NO-driven reduction of ferrihemoglobin A$_0$, 20 μM (heme), in anoxic sealed spectrophotometric cell in 50 mM bis-Tris buffer, pH 8.0 at 20°C. The reaction was initiated by the anaerobic addition of NO solution (1000 μm).

undergo oxidative modification, and the potential release of its toxic iron. The striking differences in susceptibility to oxidative damage seen with DBBF(αXL) may reflect the accessibility of its heme group to the inner-sphere oxidative attack by H_2O_2. These results also confirm earlier results which show that oxidative toxicity of hemoglobin can be experimentally manipulated irrespective of its oxygen affinity[1].

Redox Depletion of NO by Cell-free hemoglobin

Nitric oxide is now recognized to play an important role in immunology, mediation of central and peripheral nervous system functions, regulation of blood pressure and flow and in the regulation of cell activation [10,11]. Cell-free hemoglobins have been shown to produce hypertension in animals and humans [13,14]. Variable vasoconstrictive effects have been demonstrated in isolated organs perfused with variously modified hemoglobin preparations [12]. Marked hypertensive effects were recently observed with solutions of HbA_0 and DBBF(α XL) in an animal model of resuscitation from hemorrhagic shock [13]. Cell-free hemoglobins, unlike red blood cells, readily penetrate the endothelial barrier into the extravascular space where they have the potential to bind NO. This reaction, and the subsequent oxidation-reduction reactions we described here, may lead to further depletion of NO as a biological transducer and have a greater potential on the extent of vasoconstriction. Our results also show that unmodified and cross-linked hemoglobins examined exhibit somewhat variable rates at which these reactions with NO occur. These interactions of hemoglobin with NO can be enhanced or suppressed by chemical modification of the protein and/or its packaging.

Oxidative Toxicity and Nitric Oxide Interaction

Potential toxic effects of superoxide/peroxide and nitric oxide could occur as a result of reperfusion of ischemic tissues. Chemical linkage between superoxide and peroxide, both produced by reperfusion of ischemic tissues, and nitric oxide, results in the formation of yet another powerful NO interrelated redox form, peroxynitrite (ONOO$^-$) [25,26]

$$O_2^- + NO^\cdot \longrightarrow ONOO^- \qquad (8)$$

This anion, which rapidly decomposes to the highly reactive hydroxyl radical upon protonation, has been shown to oxidize sulfhydryls 1000 times faster

than H_2O_2. Modified hemoglobins, if present in close proximity to NO producing areas of the endothelium, as postulated, could serve a two fold role in a peroxynitrite cycling of NO: (1) by serving as a source of superoxide through autoxidation of the ferrous iron to the ferric form and (2) by serving as a source of protein sulfhydryls for reaction with the peroxynitrite formed.

Results reported here clearly demonstrate that the interaction of NO and activated oxygen species with cell-free hemoglobins are not necessarily directly linked to their oxygen binding delivery ability. It may be useful, therefore, to seek forms of chemical or genetic modification(s) that provide, along with desirable oxygen binding characteristics, a suppressed ability to enter into reactions that mediate free radical formation and/or nitric oxide Hb interaction.

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TOXICITY OF HEMOGLOBIN SOLUTIONS: HEMOGLOBIN IS A LIPOPOLYSACCHARIDE (LPS) BINDING PROTEIN WHICH ENHANCES LPS BIOLOGICAL ACTIVITY

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ABSTRACT

Administration of $\alpha\alpha$-crosslinked stroma-free hemoglobin (SFH) as a cell-free resuscitation fluid is associated with multiple organ toxicities. Many of these toxicities are characteristic of the pathophysiological effects of bacterial endotoxins (lipopolysaccharide, LPS). To better understand the potential role of LPS in the observed in vivo toxicities of SFH, we examined mixtures of SFH and E. coli LPS for evidence of LPS-SFH complex formation. LPS-SFH complexes were demonstrated by three techniques: ultrafiltration through 300 kDa cut-off membranes, which distinguished LPS in complexes (87-89% <300 kDa) from LPS alone (90% >300 kDa); density centrifugation through 5% sucrose, which distinguished denser LPS alone from LPS-SFH complexes; and precipitation by 67% ethanol, which demonstrated 2-3 fold increased precipitability of complexes compared to SFH alone. Interaction of LPS with SFH was also associated with markedly increased biological activity of LPS, as manifested by enhancement of

LPS activation of Limulus amebocyte lysate (LAL), increased release of human mononuclear cell tissue factor, and enhanced production of cultured human endothelial cell tissue factor. These results demonstrated that hemoglobin can serve as an endotoxin binding protein, and that this interaction results in the alteration of several LPS physical characteristics and enhancement of LPS biological activities.

INTRODUCTION

Stroma-free hemoglobin, a preparation of purified human hemoglobin, is being developed for use as a cell-free resuscitation fluid [1-3]. In order to stabilize the protein's tetrameric structure, preparations of stroma-free hemoglobin have been covalently crosslinked between the protein chains. αα-crosslinked stroma-free hemoglobin (SFH) is a modified hemoglobin, crosslinked between the α chains with bis(3,5-dibromosalicyl) fumarate, that demonstrates prolonged in vivo retention. SFH has excellent oxygen binding and delivery properties, as well as an adequate half-life, and therefore is a potentially ideal "blood substitute". However, in vivo administration of SFH has revealed significant problems of toxicity, including hypertension and bradycardia [4,5], a decrease in glomerular filtration rate and renal plasma flow [6], mild prolongations of the partial thromboplastin time [5] and fever. In some studies, administration of SFH has resulted in activation of the complement and coagulation cascades [7-9], disseminated intravascular coagulation with resultant thrombosis [7,10,11], and ischemic parenchymal damage [7,8].

Many of the reported toxicities of SFH infusion can be explained by the known consequences of endotoxemia, and the presence of LPS in preparations of SFH utilized for in vivo studies has been documented [7,9]. Therefore, a contributory role for the observed in vivo toxicity of SFH has been proposed for bacterial endotoxin [10,11]. Previously, SFH and LPS have been shown to produce synergistic in vivo toxicity [11], and we have demonstrated that SFH is capable of enhancing the procoagulant activity of LPS in vitro [12]. We hypothesized that SFH

binds LPS, and that the interaction between these molecules could alter the biological activity of LPS. The present study was designed to determine whether complex formation occurs between SFH and LPS, and evaluate the ability of SFH to alter biologic activities of LPS.

MATERIALS AND METHODS

Reagents. Sterile, 15 ml Falcon tubes were obtained from Becton Dickinson (Mountainview, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL).

Glassware. Glassware was heated at 190°C in a dry oven for 4 hours.

Hemoglobin. Human SFH, crosslinked between α chains with bis(3,5-dibromosalicyl)fumarate as described previously [13,14], was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA. SFH was 9.6 g/dl, pH 7.4, 95.4% crosslinked, 96.3% oxyhemoglobin, 3.2% methemoglobin, and contained less than 0.4 EU/ml endotoxin (referenced to E. coli lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test [15]. The SFH stock solution was stored at -70°C , and then diluted with sterile, pyrogen-free 0.9% NaCl prior to use. Carboxy-SFH(CO-SFH), produced by incubation of the SFH solution with CO, was at 9.6 g/dl, 95.4% crosslinked, 95% HbCO and 5% oxyhemoglobin. Purified non-crosslinked human A_0, 8.4 g/dl, also provided by collaborators at BRD/LAIR, was prepared by ion exchange HPLC of purified human hemoglobin, as described previously [16].

Endotoxin. E. coli O26:B6 lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). ^{14}C-LPS (Salmonella typhimurium PR122(Rc)) was purchased from List Biologicals, Inc. (Campbell, CA) and was resuspended in endotoxin-free water at 1 μCi/ml (1 mg/ml).

Limulus amebocyte lysate (LAL). Amebocyte lysates were prepared from Limulus polyphemus (the North American horseshoe crab) by disruption of washed amebocytes in distilled water [15,17].

Chromogenic Substrate. Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger.

Chromogenic Limulus Amebocyte Lysate (LAL) Test. 50 µl of sample
and 30 µl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were
incubated in tissue culture plates for 30 min at 37°C in a temperature-
controlled plate reader (Kinetic-QLC, Whittaker Bioproducts Inc.,
Walkersville, MD). 40 µl chromogenic substrate S-2423 (0.25 mM, in 25
mM Tris, pH 8.6) was added to each well, mixtures were incubated at
37°C for 5 min, and absorbances at 405 nm then were determined.

Ultrafiltration. Solutions of SFH, CO-SFH or A_0 were prefiltered
through an endotoxin-free 300 kDa membrane prior to use to remove
aggregated protein particles. SFH, CO-SFH or A_0 (100 µg/ml) was
incubated with E. coli O26:B6 (W) LPS (50 µg/ml) for 30 min at 37°C.
Mixtures then were filtered manually with a 3 ml syringe (according to
the directions of the filter manufacturer) at room temperature, using a
300 kDa cut-off filter (ultrafree-PFL polysulfone 300, Millipore
Corporation, Bedford, MA). LPS concentrations in filtered solutions of
hemoglobin, hemoglobin and LPS mixtures, or LPS alone were
determined by the chromogenic LAL test (described above), using
starting mixtures of hemoglobin-LPS, or LPS alone, for the standard
curve. Hemoglobin protein concentrations were determined by the BCA
protein assay (Pierce, Rockford, IL).

Sucrose centrifugation of LPS and SFH. ^{14}C-S. typhimurium LPS
(0.005 µCi) was added to each of the hemoglobin solutions (each diluted
to 10 mg/ml), and the mixtures were incubated for 30 min at 20°C.
Aliquots of LPS-hemoglobin mixtures, LPS alone, or hemoglobin alone
then were layered over 5% pyrogen-free sucrose and centrifuged at
2,900 x g for 30 min at 20°C, in a Sorvall RC-5 centrifuge (Du Pont
Instruments, Wilmington, DE). Scintillation counting was performed,
after samples were diluted 10-fold in fluor (Formula A-989, NEN
Research Products, Boston, MA), in a Tracor Analytic Liquid
Scintillation System (Tracor Analytic, Elk Grove Village, IL). For
samples containing hemoglobin, quenching of ^{14}C-LPS by hemoglobin
was reversed as follows: 0.1 ml aliquots of fractions were diluted ten-
fold in water (to 1 ml final volume), and 1 ml Solvable (NEN Research
Products, Boston, MA) was added. These mixtures were incubated at
60°C for one hr, and then 0.3 ml 25% H_2O_2 was added.

Ethanol precipitation of SFH and LPS-SFH mixtures. 2 µg SFH, CO-SFH, or A_0 was incubated with 25 µg E. coli O26:B6 (W) LPS in microtiter plate wells for 30 min at 20°C. Ethanol then was added to each well (final concentration, 67%), and after an additional 30 min the mixtures were centrifuged at 800 x g for 30 min. The concentrations of hemoglobin in the sediments were determined by protein assay, and LPS concentrations by the phenol-concentrated H_2SO_4 method [18].

Mononuclear cell (MNC) tissue factor (TF) assay. E. coli LPS (100 ng/ml) was incubated with SFH (6 mg/ml) for 30 min at 37°C. LPS alone, SFH alone, or LPS-SFH mixtures were then incubated for 20 hr at 37°C with human peripheral blood MNC [19] and assayed for TF with a one-stage coagulation assay [20]. A clotting time of 30 sec was defined as equal to 100 units TF activity [21].

Endothelial cell tissue factor (TF) assay. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured in media (containing 2% serum) obtained from Clonetics. Cells were grown to confluent monolayers in sterile 96-well microtiter plate wells (Nunclon, Applied Scientific, San Francisco). E. coli LPS alone, SFH alone, or LPS-SFH mixtures were added to the media in each well (final concentrations: 1 µg/ml LPS; 1 mg/ml SFH), and incubated for 4 hr. Wells were then washed with media (x 3) and the HUVEC were freeze-thawed (x 2) and sonicated in phosphate buffered saline. To each well then was added normal human citrated plasma and calcium (25 mM), and plates were incubated for 8 min in a temperature-controlled (37°C) plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). Turbidity was measured at 340 nm, and TF activity was calculated from a standard curve established with rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 min by 1:100 diluted thromboplastin was arbitrarily defined as 10 TF units.

RESULTS

Demonstration of LPS-SFH complexes. Ultrafiltration experiments demonstrated that 87-89% of the LPS in LPS-SFH mixtures was

filtered through the 300 kDa membrane, whereas only 10% of LPS alone was filterable (Fig. 1). This indicated that SFH caused the dissociation of LPS into lower molecular weight particles. Approximately 90% of the total SFH protein in each of the three LPS-SFH mixtures, and from filtrates of SFH alone, was detected in filtrates (data not shown). Utilizing ethanol precipitation, greater than twice the amount of each SFH was precipitated in the presence of LPS than was with SFH alone (Fig. 2). In both the absence and presence of SFH, approximately 90% of LPS was precipitated by ethanol (data not shown). Following sucrose centrifugation, 76% of LPS sedimented into the bottom fraction in the absence of protein, whereas only 3-9% sedimented in the presence of any of the three SFH preparations (Figure 3). Conversely, only 3% of LPS alone remained above the sucrose layer, whereas in the presence of SFH, 64-79% of LPS remained in the top layer. No detectable SFH entered the sucrose layer in either the absence or presence of LPS. Therefore, SFH decreased the density of LPS, resulting in the co-migration of SFH and LPS.

Biological activity of LPS in SFH-LPS complexes. SFH increased the biological activity of LPS in three independent assays. First, LPS in the presence of SFH produced enhanced activation of LAL (3 to 4.5-fold) compared to LAL activation by LPS alone (Fig. 4). Second, LPS-SFH complexes resulted in 5.5-fold greater TF production by human MNC than the TF generated from MNC by LPS alone (Fig. 5). Third, SFH resulted in a 2.8-fold increase in endothelial cell TF production compared to TF generated by LPS alone (Fig. 6).

DISCUSSION

We performed experiments to determine whether SFH interacted with LPS. Ultrafiltration demonstrated that the molecular weight of LPS (typically $>10^6$ in aqueous solution) was reduced to < 300 kDa in the presence of SFH, and that LPS and SFH co-filtered. Utilizing centrifugation through sucrose, we showed that the density of LPS in the presence of SFH was distinctly less than that of LPS alone, and that LPS and SFH co-migrated. Measurement of SFH precipitation by
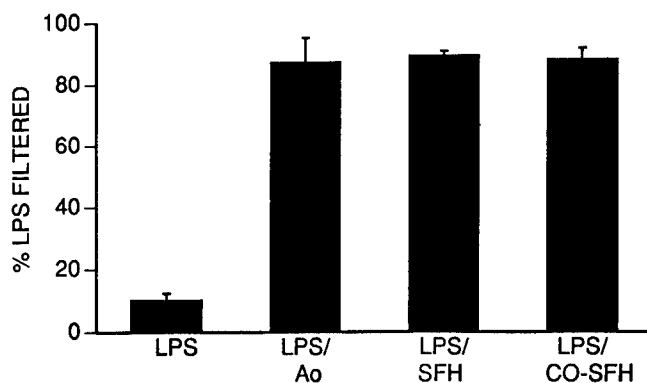
FIGURE 1. Ultrafiltration of SFH and LPS. E. coli LPS was incubated with SFH, CO-SFH or A_0, and the mixtures were filtered through a 300 kDa cut-off ultrafiltration membrane. The % of LPS filtered was deter-mined by the LAL test. All three preparations of SFH greatly increased the filterability of LPS.
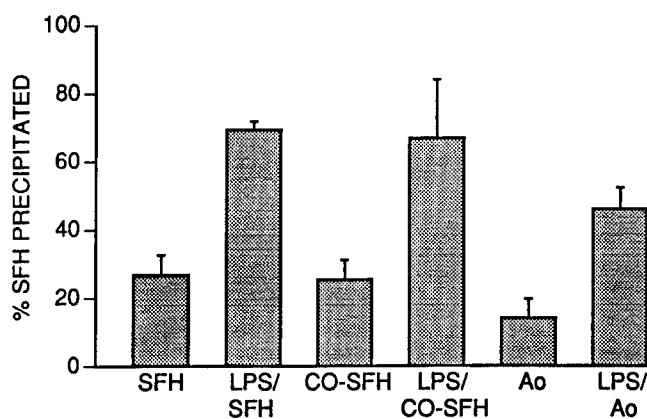
FIGURE 2. Precipitation of SFH and LPS by ethanol. E. coli LPS was incubated with SFH, CO-SFH, or A_0, and the LPS-SFH complexes were then precipitat-ed from the mixtures by 67% ethanol and sedimented by cen-trifugation. Each preparation of SFH demonstrated increased precipitability in the presence of LPS.
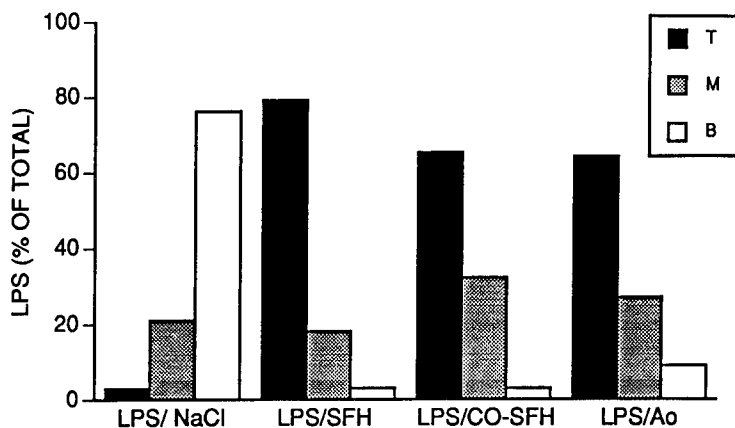
FIGURE 3. Centrifugation of SFH and LPS through sucrose. [14]C S. typhimurium LPS was incubated with SFH, CO-SFH, or A$_0$, and the mixtures were then centrifuged through 5% sucrose. The distributions of radiolabeled LPS were determined in top (T), middle (M) and bottom (B) zones of the centrifuged samples. All three preparations of SFH co-migrated with LPS, resulting in a decrease in LPS density.
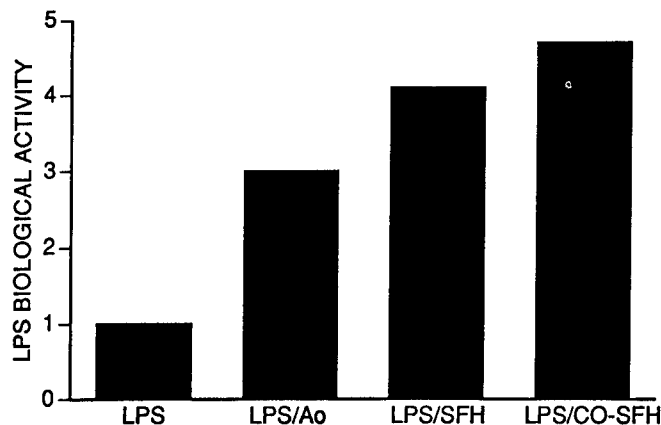
FIGURE 4. Effect of SFH on LPS biological activity in the LAL test. E. coli LPS, in the absence or presence of SFH, CO-SFH, or A$_0$ was incubated with LAL, and activation measured with a chromogenic substrate. LPS biological activities in LPS-protein mixtures are expressed as relative activities to LPS alone. All three preparations of SFH resulted in increased biological activity of LPS.
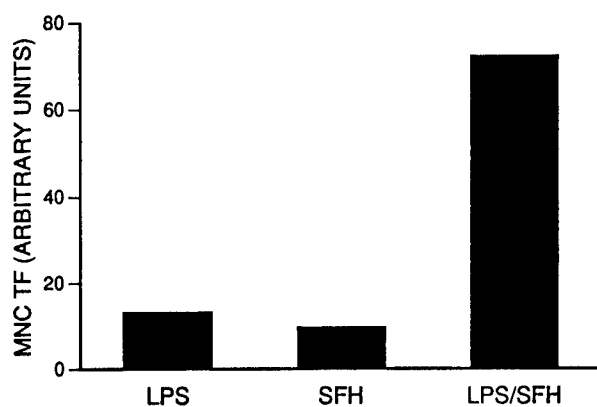
FIGURE 5. Effect of SFH on the LPS-induced stimulation of TF activity in human MNC. E. coli LPS, in the absence or presence of SFH, was incubated with human MNC, and TF activity was measured with a plasma clotting assay. SFH enhanced the ability of LPS to stimulate TF production.
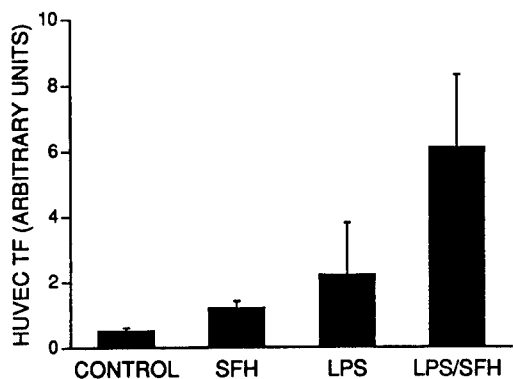
FIGURE 6. Effect of SFH on the LPS-induced stimulation of TF activity in HUVEC. E. coli LPS, in the absence or presence of SFH, was incubated with HUVEC, and TF activity was measured with a plasma clotting assay. SFH enhanced the LPS-induced production of TF from the endothelial cells.

ethanol indicated that LPS greatly increased the precipitability of SFH. Therefore, our experiments demonstrated that the physical characteristics of both SFH and LPS were altered in LPS-SFH mixtures. These results are consistent with the formation of stable complexes, and establish the ability of hemoglobin to act as an endotoxin-binding protein. Because these results were observed with unmodified hemoglobin (A_0) and CO-SFH (which was not susceptible to methemoglobin production), as well as with SFH, we have demonstrated that LPS-binding is an intrinsic property of hemoglobin.

The formation of LPS-SFH complexes was associated with major changes in the procoagulant activities of LPS. SFH enhanced the ability of LPS to stimulate coagulation via three independent mechanisms: 1) direct activation of the proteolytic coagulation cascade of Limulus, 2) stimulation of TF production from human MNC, and 3) stimulation of TF production from HUVEC. Enhancement by SFH of LPS procoagulant activity may contribute to the observed thrombosis and ischemic damage associated with SFH infusion in animals [7,8], and may also provide a mechanism for the synergistic toxicity between SFH and LPS reported previously [11,22]. Interestingly, other proteins that are known to bind LPS with a resultant change in LPS biological activity (e.g., mellitin [23], lysozyme [24], and complement [25] or the polypeptide polymyxin B [26]) cause a decrease in LPS toxicity.

Our observations that LPS, when complexed with SFH, was of much lower molecular weight and lesser density than LPS alone suggest that SFH caused the disaggregation of LPS. In contrast to the increased biological activity we observed for LPS that had been disaggregated and bound to SFH, the process of LPS disaggregation in plasma (resulting primarily from its interaction with high density lipoproteins [27]), results in detoxification. It is possible that the process of LPS-SFH complex formation might potentially interfere with LPS detoxification in plasma.

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ASSESSMENT OF HEMOGLOBIN-DEPENDENT NEUROTOXICITY: ALPHA-ALPHA CROSSLINKED HEMOGLOBIN

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ABSTRACT

Adult human hemoglobin A_0 (HbA$_0$) has been shown to be neurotoxic, and we wish to report on similar studies conducted using a modified hemoglobin, which has been crosslinked between the alpha subunits (α–αHb). Cortical cell cultures were prepared from fetal Swiss-Webster mice at 15-16 days gestation. Mature cultures (days *in vitro*, 12-16) were exposed to α–αHb in a defined medium for 24-48 hours at 37° C. Low micromolar amounts of α–αHb were neurotoxic in a concentration-dependent fashion. This toxicity was attenuated by the antioxidants Trolox and U-74500A and by the iron chelator deferoxamine. The hemoglobin-binding protein, haptoglobin, also completely blocked α–αHb-dependent neurotoxicity. The latter result was unexpected because complex formation between α–αHb and haptoglobin was not detected using assays of haptoglobin fluorescence and hemoglobin peroxidase activity.

INTRODUCTION

When vascular components are extravasated, the surrounding tissues are exposed to cellular and molecular moieties that may be toxic. One such

component is the erythrocyte, the lysate of which contains significant quantities of hemoglobin, which may be problematic in the central nervous system (CNS). Hemoglobin is thought to be the primary causative agent of cerebral vasospasm, a common complication of hemorrhagic events in the CNS [1]. Small amounts of hemoglobin, when injected into or applied to the surface of the cerebral cortex, result in electrical as well as behavioral epileptiform activity within 48 hours [2,3,4].

In addition, hemoglobin-bound iron can serve as a pro-oxidant, catalyzing the formation of oxygen radicals that are capable of damaging cellular lipids, protein, and nucleic acids. The CNS is inordinately susceptible to this type of injury because it: (1) has high concentrations of polyunsaturated fatty acids that are oxidized easily; (2) contains high concentrations of ascorbate that can cycle iron between its oxidized and reduced state, increasing the toxicity of iron; (3) is not well endowed with antioxidant protective enzymes; and (4) is post-mitotic and not capable of regeneration following injury [5]. Therefore, we hypothesized that cultures of cortical neurons would exhibit toxicity upon exposure to solutions of hemoglobin. This possibility was confirmed using chromatographically-purified adult human hemoglobin A_0 (HbA$_0$) [6]. The experiments of the current study were designed to assess the effectiveness of a modified hemoglobin, covalently crosslinked between the alpha subunits, as a pro-oxidant in cortical neuronal cultures.

METHODS AND MATERIALS

All hemoglobin solutions were prepared in the pilot-plant production facility at the Letterman Army Institute of Research. Human A_0 hemoglobin was purified by high pressure liquid chromatography according to previously published techniques. [7,8]. Hemoglobin concentrations were determined using Drabkin's solution [9] and methemoglobin concentrations were determined to be less than 4.0% according to the method of Evelyn and Malloy [10,11]. All hemoglobin concentrations are expressed as the concentration of heme, or hemoglobin

monomer. Hemoglobin crosslinked between the alpha subunits with bis(3,5-dibromosalicyl)fumarate (DBBF) was produced according to the method of Winslow and Chapman [12].

Cortical cell cultures were prepared with modification of methods described by Choi et al. [13]. After halothane anesthesia, pregnant Swiss-Webster mice (15-16 days gestation) were sacrificed by cervical dislocation. The neocortex was then dissected and incubated in media containing 0.09% acetylated trypsin at 37°C for one hour. Tissue was then disassociated by trituration through a flame-polished Pasteur pipette in plating media consisting of Eagle's minimal essential medium (MEM), 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM), and glucose (21 mM). The cell suspension was then diluted in plating media and plated on glial cultures in 15 mm multiwell plates (2.2 hemispheres/plate). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and the media was partially changed twice weekly with media identical to plating media but lacking fetal serum. Non-neuronal cell division was inhibited at 6-9 days *in vitro* by addition of 10^{-5} M cytosine arabinoside. Cortical glial cultures were prepared in a similar fashion from neonatal Swiss-Webster mice, except that plating media contained 10% fetal bovine serum, 10% horse serum, and epidermal growth factor (10 ng/ml).

Cultures were exposed to α–αHb at 12-15 days *in vitro*. At this time, neurons were easily distinguished from the background layer of astrocytes by their phase-bright cell bodies and extensive network of processes. Cultures were washed free of culture media and placed into MEM. After addition of α–αHb-containing solutions, cultures were incubated at 37°C in a 5% CO_2 atmosphere. Neuronal injury was estimated by examination of cultures with phase-contrast microscopy and was quantified by measurement of lactate dehydrogenase (LDH) in the culture media at the end of the exposure period [14]. The assessment of neuronal death by LDH release correlates well ($r^2 = 0.90$-.95) with the quantitation of cellular death by actual cell counts [14, and personal communication, R. Regan].

The binding of hemoglobin to haptoglobin was measured spectrofluorometrically using the technique of Nagel and Gibson [15], as previously reported [16]. In short, the excitation wavelength was 284 nm and emission wavelength was 350 nm, and all assays were conducted in 10.0 mM phosphate buffer, pH 8.0, at 25° C. The concentration of hemoglobin was 33 nM (heme), and the concentration of haptoglobin was 90 nM, based on a molecular weight of 110 Kd. The peroxidase activity of hemoglobin and the haptoglobin-hemoglobin complexes, using 2,2'-azino-bis(3-ethylbenz-thiazolinesulfonic acid) (ABTS) as substrate was measured using the methods of Everse [17]. In brief, reactions were conducted in phosphate-citrate buffer (50 mM, pH 5.4) with 0.91 mM ABTS. The reaction was started by the addition of 0.1 ml 176 mM hydrogen peroxide and monitored at 740 nm.

RESULTS

Exposure of cultures to α–αHb for 24-28 hours resulted in a concentration-dependent neuronal injury; a hemoglobin concentration of approximately 3.0 μM produced a 50% effect (EC50) (Figures 1 and 2b). After a 24 hour exposure to 100 μM hemoglobin, over 80% of the neurons had degenerated. In contrast, the background layer of glia sustained no apparent injury.

Experiments performed with purified HbA_0 demonstrated that neurons could be protected from hemoglobin-dependent toxicity by the antioxidants Trolox (a water soluble analog of α-tocopherol, Aldrich Chemical Co.) and U74500A (The Upjohn Company) and the iron chelator/antioxidant deferoxamine (Sigma Chemical Co.) [6], and similar experiments were performed with α–α crosslinked hemoglobin. When hemoglobin incubations were performed in the presence of the antioxidant U74500A (1 μM), the neurons appeared to be as healthy as those observed in control cultures (Figures 2c and 3). The same results were observed when cultures were exposed to α–αHb in the presence of deferoxamine
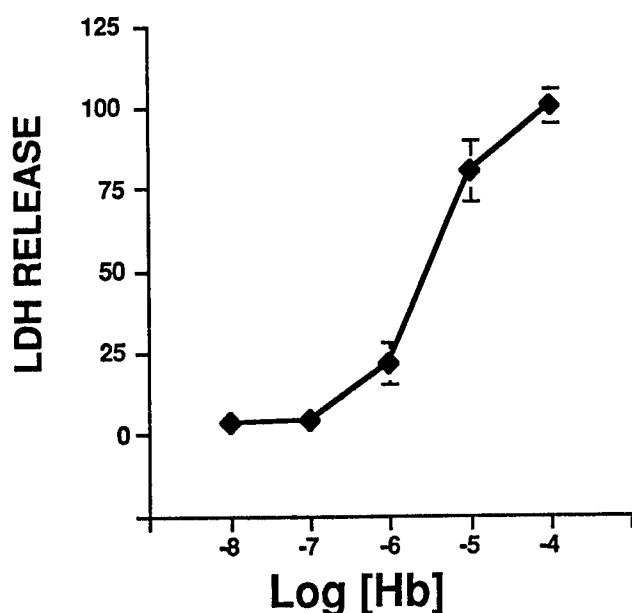
Figure 1.   Alpha-alpha crosslinked human hemoglobin is toxic to cortical neurons in culture. Cultures were exposed to hemoglobin for 24 hours, at which time a sample of the culture media was removed and assayed for LDH activity. All data were scaled to the LDH released by the highest concentration of hemoglobin (100 μM, equals 100, which represents the death of approximately 80% of all neurons). Values represent means ± SEM.

(100 μM) (photomicrograph not shown). U74500A and deferoxamine were neuroprotective at 1 and 10 μM, respectively (Figure 3). Significant neuroprotection was provided by 10.0 μM Trolox.

Prior experiments conducted with HbA_0 demonstrated that hemoglobin-dependent neurotoxicity could be blocked by pre-mixing the hemoglobin with purified human haptoglobin type 1-1 (Sigma Chemical Co.) (data not shown). To evaluate the ability of haptoglobin to block α–αHb neurotoxicity, experiments were designed to utilize the natural ability of haptoglobin to stabilize heme and heme-iron in hemoglobin. Complete
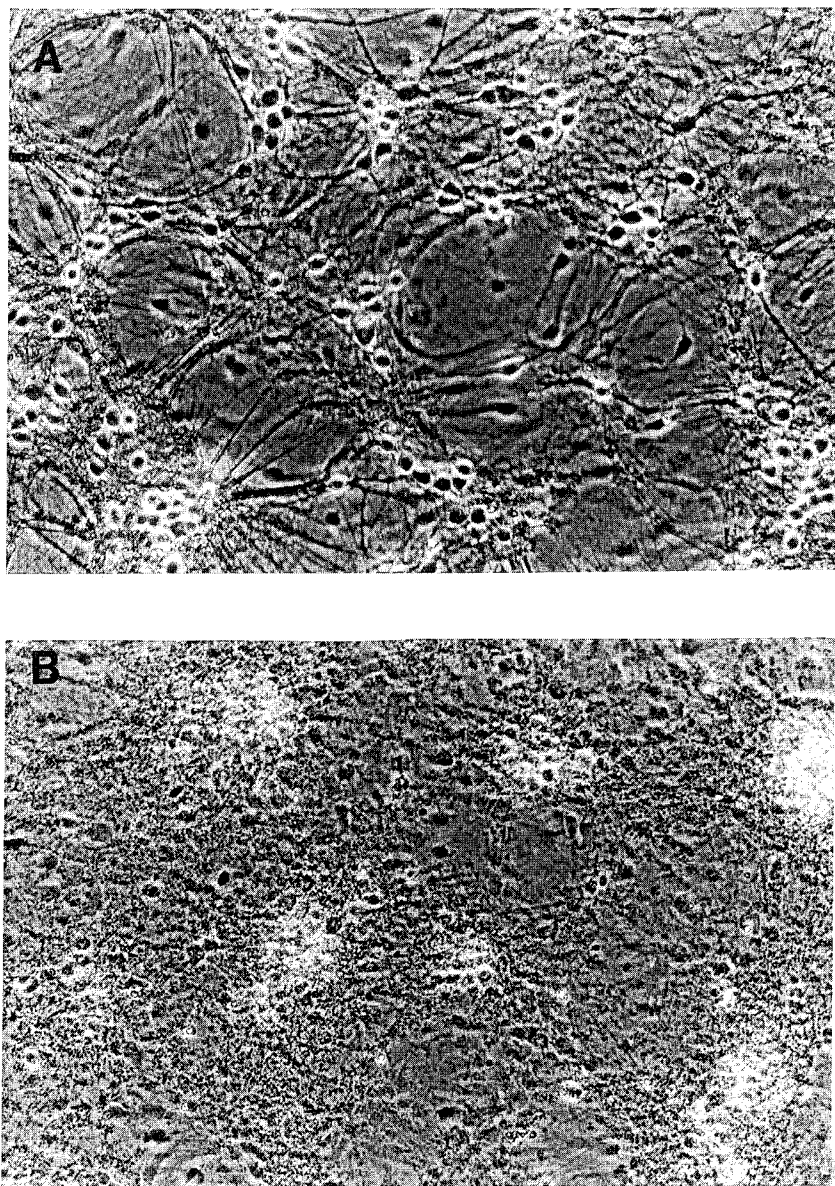
Figure 2.   Phase contrast photomicrograph of neuronal cultures exposed to α–α
crosslinked hemoglobin. Panel (a) is a control culture that was exposed to all washes and
MEM; panel (b) was exposed to 100 μM α–αHb; and panel (c) was exposed to 100 μM α–
αHb in the presence of 1.0 μM U74500A.
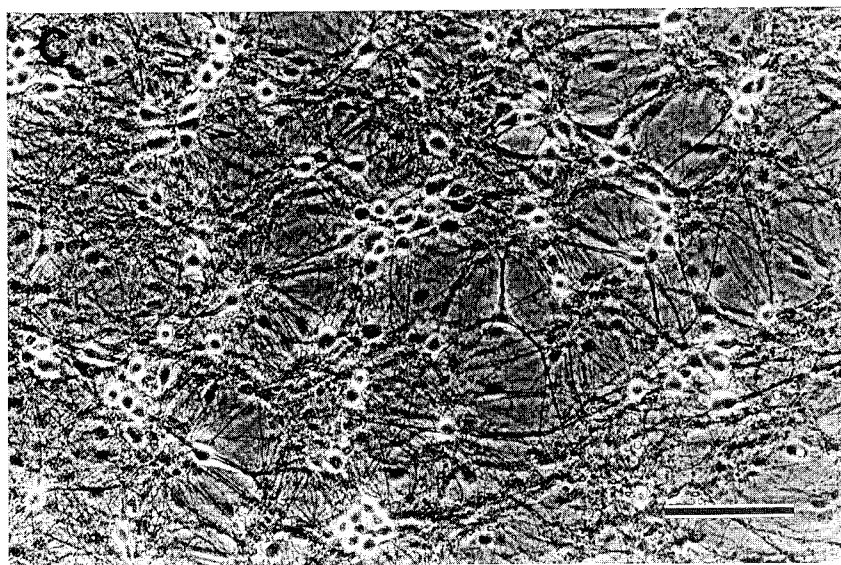
Figure 2. Continued

protection from α–αHb neurotoxicity was provided by haptoglobin at a molar ratio of 1.0:1.6 hemoglobin: haptoglobin (Figure 4). The difference in neurotoxicity between α–αHb and HbA$_0$ was not statistically significant, but it reflects a consistent trend.

To assess the interaction of α–α crosslinked Hb with human haptoglobin, binding experiments were conducted with HbA$_0$, α–αHb, and a 50:50 mixture of HbA$_0$:α–αHb. HbA$_0$ quenched Hp fluorescence > 60% in two minutes (Figure 5). Alpha-alpha crosslinked Hb, on the other hand, quenched at a rate equivalent to that of the baseline quench of Hp alone. The mixture of HbA$_0$ and α–αHb quenched Hp fluorescence to a degree that would be expected if there were no interaction between α–αHb and Hp. A second assessment of haptoglobin binding was performed by measuring the peroxidase activity of haptoglobin-hemoglobin complexes
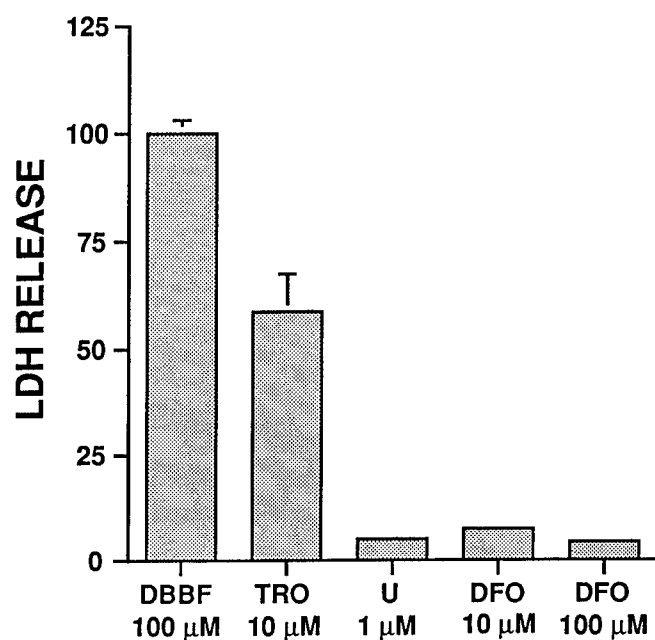
Figure 3.   Pharmacology of α–αHb-dependent neurotoxicity. Cultures were exposed to 100 μM α–αHb (DBBF) in the presence and absence of Trolox (TRO), U74500A (U), or deferoxamine (DFO) for 24 hours. Data were scaled to the LDH released by 100 μM α–αHb (equals 100). Values represent means ± SEM.

compared with that of hemoglobin alone. In the presence of haptoglobin, the peroxidase activity of HbA_0 increases (Figure 6), but there was no haptoglobin-related increase in the peroxidase activity of α–αHb. Peroxidase activity of α–αHb was greater than that of HbA_0 in either the presence or absence of Hp.

DISCUSSION

These results suggest that α–αHb is highly toxic to neurons in culture, which agrees with previous demonstrations of Hb-dependent neuronal
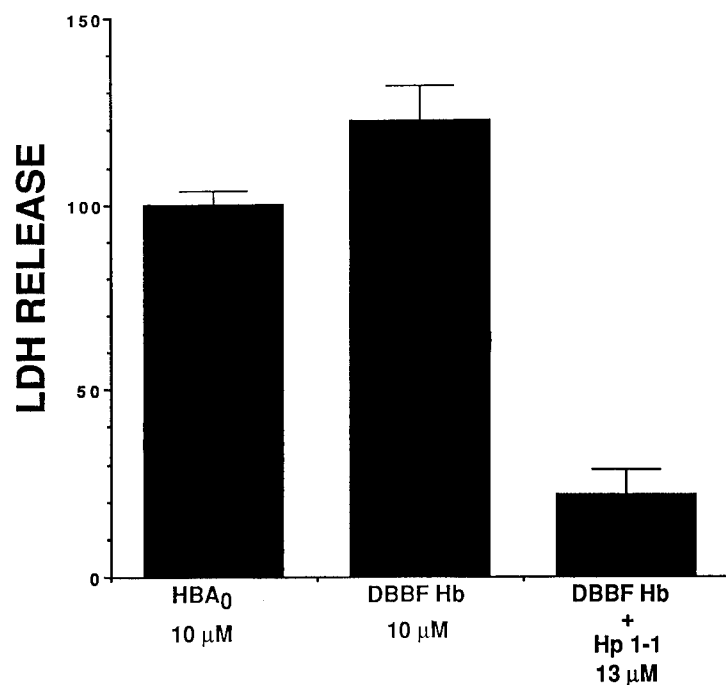
Figure 4.   Human haptoglobin type 1-1 protects agains α–αHb (DBBF Hb)-
dependent neurotoxicity. Neuronal cultures were exposed to 10 μM α–αHb for 48 hours
in the presence and absence of 16 μM human haptoglobin type 1-1. Data were scaled to
the LDH released by 100 μM HbA$_0$ (equals 100). Values represent means ± SEM.

injury *in vivo* [3,4,18] and *in vitro* [6] and in biochemical studies of
hemoglobin-dependent lipid injury [18,19,20,21,22]. The pharmacology
of α–α-Hb-dependent neurotoxicity agrees well with similar experiments
performed with HbA$_0$ [6].

The ability of antioxidants to inhibit neural injury suggests that hemoglobin
mediates its toxic effect via oxidant mechanisms, and the dependence upon
iron was confirmed by the ability of low micromolar concentrations of
deferoxamine to inhibit neural injury. If deferoxamine were functioning
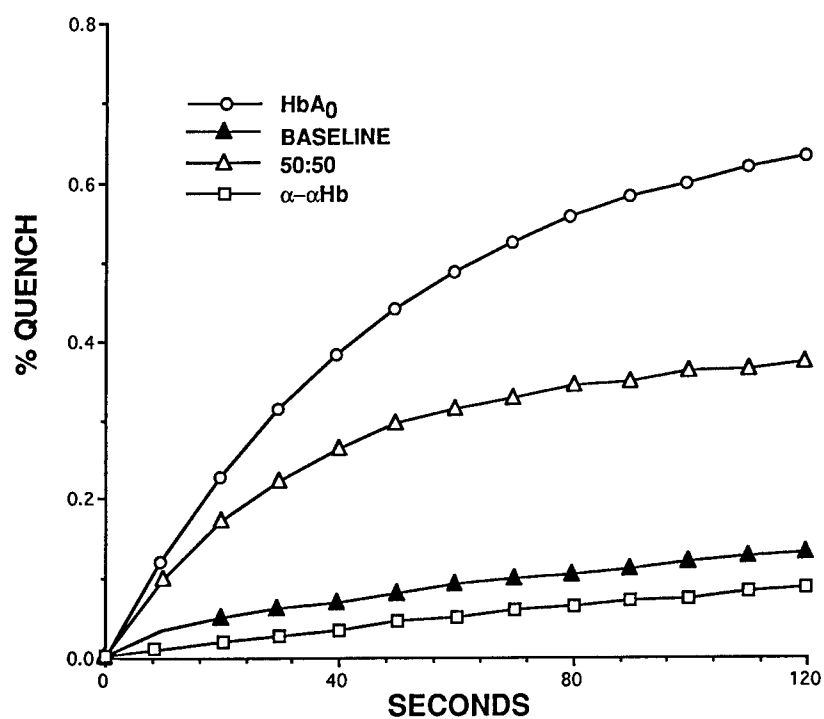
Figure 5.   The quenching of haptoglobin fluorescence by HbA0 and α–αHb.
Excitation wavelength was 284 nm and emission wavelength was 350 nm. Assays were
conducted in 1.0 mM phosphate buffer, pH 8.0, at 25° C. The concentration of both
hemoglobins was 33 nM (heme), and the concentration of haptoglobin was 90 nM, based
on a molecular weight of 110 Kd.

more as a scavenger than a chelator, inhibition would not occur until
millimolar concentrations were utilized. In this cell culture system, similar
neurotoxicity was observed with both HbA0 and α–αHb.   In contrast,
HbA0 has been found to be more potent than α–αHb in catalyzing free
radical reactions in a cell free system [23]. In our experimental conditions,
this difference is apparently not of sufficient magnitude to influence
neuronal injury.
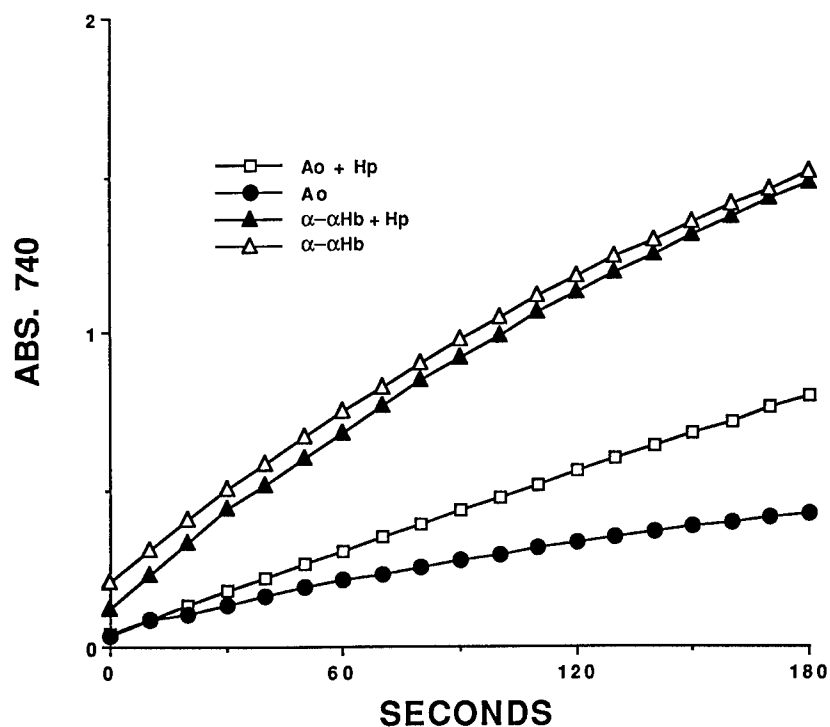
Figure 6.   The peroxidase activity of HbA$_0$ and α–αHb in the presence and absence of human haptoglobin type 1-1. Reactions were conducted in phosphate-citrate buffer (50 mM, pH 5.4) with 0.91 mM 2,2'-azino-bis(3-ethylbenz-thiazolinesulfonic acid) (ABTS) as substrate. The reaction was started by the addition of 0.1 ml 176 mM hydrogen peroxide and monitored at 740 nm.

Previous biochemical experiments *in vitro* had indicated that α–αHb did not bind to haptoglobin as assessed by monitoring the decrease in haptoglobin fluorescence upon mixing with hemoglobin [16]. In the current study, the change in hemoglobin-dependent peroxidase activity in the presence of haptoglobin was not observed with α–αHb, once again suggesting that haptoglobin did not bind α–αHb. The peroxidase activity of α–αHb itself, however, was much higher than that exhibited by either

HbA$_0$ alone or HbA$_0$-haptoglobin complexes. These findings agree with the conclusions of Alayash et al., who found that structural modifications of hemoglobin alter the redox potential of hemoglobin-bound iron [23]. The results of the two previous experiments were consistent with the hypothesis that α–αHb and human haptoglobin do not interact. However, the complete neuroprotection provided by haptoglobin type 1-1 suggests that, under our experimental conditions, an interaction between α–αHb and haptoglobin occurs but does not affect Hp fluorescence or alter α–αHb peroxidase activity.

An interaction between α–αHb and haptoglobin has not been described previously . The ramifications of the hemoglobin-haptoglobin interaction are important to normal physiology. Free intravascular or extravascular hemoglobin is normally cleared by the liver as hemoglobin-haptoglobin complexes that are recognized by specific hepatic receptors [24]. If one hypothesizes that a hemoglobin-based oxygen carrier (HBOC) will form a haptoglobin complex that is recognized by the hepatic receptors, plasma haptoglobin concentrations will be immediately and severely depleted upon initial administration of the HBOC. Consequently, haptoglobin will not be available to assist in the clearance of extravasated endogenous hemoglobin. Whether or not a complex forms between α–αHb and Hp, hemoglobin clearance mechanisms will not occur through normal pathways, and the pharmacokinetics of HBOC's may involve body compartments that do not normally have contact with free hemoglobin, possibly resulting in toxicity. Additionally, there will be no plasma haptoglobin to serve as an antioxidant by stabilizing extravasated hemoglobin.

In conclusion, α–αHb is toxic to neurons in culture, possibly through oxidant mechanisms. The ability of haptoglobin to inhibit α–αHb neuronal inury suggests that these two molecules may interact in a previously undescribed fashion.

ACKNOWLEDGMENTS

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CROSS-LINKING HEMOGLOBIN BY DESIGN: LESSONS FROM USING MOLECULAR CLAMPS

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ABSTRACT

The development of a red cell substitute by chemical modification of hemoglobin has been approached as a systematic, iterative process. Acyl phosphate methyl esters were designed as anionic electrophiles to permit selective acylation of amino groups in the cationic site of hemoglobin which binds polyanions. Kinetic studies with systematically substituted acyl phosphates and amines show that the reaction is controlled by a reversible addition step followed by an irreversible elimination step. Acyl phosphate methyl esters which are derivatives of rigid dicarboxylic acids introduce cross-links in human hemoglobin between amino groups in the β subunits (ε-NH$_2$-Lys-82, α-NH$_2$-Val-1) and permit correlation of oxygen binding properties with cross-link structure. The data suggest that the cross-link maintains cooperativity while reducing overall oxygen affinity by lowering the affinity of the R form for oxygen rather than by perturbing the R,T equilibrium of native hemoglobin. Materials produced from deoxyhemoglobin with a cross-link between positions 1 and 82 of the two β units

have appropriate oxygen affinity for red cell substitutes. The use of a trifunctional cross-linker, trimesyl tris(methyl phosphate) selectively produces hemoglobin with the desired 1-82 connection in good yield. The reagent is readily prepared and the properties of this chemically modified hemoglobin are suitable for trial as a red cell substitute, closely resembling those of optimized materials produced by recombinant technology. Further work is producing new chemicals and providing structural information.

INTRODUCTION

Through an ongoing collaboration involving cycles of structural analysis, chemical design, chemical synthesis, hemoglobin modification, and physical measurements, we have developed methods for the efficient production of a modified human hemoglobin which has properties expected to be useful as an oxygen carrier in a blood substitute. Other workers have used genetic engineering in bacterial expression systems with feedback cycles in which the properties of the cloned and expressed product were systematically improved. The structural features and properties of our chemically modified hemoglobin ("Triply-linked") [1] and the genetically engineered material ("RHb 1.1") [2] converged toward the same goal. The modified hemoglobins have very different structural modifications. Yet, the properties related to oxygen delivery are almost identical and significantly different from native hemoglobin.

TABLE I

25°C, pH 7.7	Native, Hb A	Triply-linked	RHb 1.1
Bohr coefficient	-0.47	-0.32	-0.32
P50	4.5	18.1	17.2
Hill coefficient	2.9	2.6	2.35
Alpha chain	native	native	alpha-gly-alpha
Alpha N-terminus	Val Leu	Val Leu	Met Leu
Beta Chain-108	Asn	Asn	Lys
Beta N-terminus	Val His	Val His	Met His

These results show that the design and production of a red cell substitute must cross the boundaries of traditional scientific disciplines. Insights are needed from chemistry, biochemistry, physiology and medicine, providing signifcant opportunities and rewards for collaborative efforts.

SOME CHEMICALS WHICH GIVE USEFUL MODIFIED HEMOGLOBINS

The development of chemicals for the modification of hemoglobin to produce a blood substitute started by focussing on the effects of a selected chemical in modifying hemoglobin.   For example, Walder and co-workers have developed a chemically cross-linked material, HbXL99α, from the reaction of deoxyhemoglobin with fumaryl bis(3,5-dibromosalicylate) in the presence of a polyanion [3,4]. Their work has optimized the use of this material in producing a red cell substitute from hemoglobin. The recent review by Vandegriff and Winslow gives an excellent perspective of the development of that material [5].

Benesch and Benesch had shown that pyridoxal phosphate can be reduced with sodium borohydride onto amino groups in the diphosphoglycerate binding site of hemoglobin [6]. This modified hemoglobin has a low oxygen affinity and they reasoned that the phosphate group was in effect a permanently installed analogue of diphosphoglycerate [7]. Pocker had designed norformyl pyridoxal phosphate (nFPLP) to cross-link proteins which bind pyridoxal phosphate [8]. Benesch and Benesch used nFPLP to cross-link hemoglobin in the diphosphoglycerate binding site, producing a stabilized tetramer with low oxygen affinity [9,10]. At least one of the products has the properties desired in a blood substitute. Since the cross-linker is extremely difficult and costly to prepare it has not yet been used for a clinical product.   By comparison, Walder's route to HbXL99α is a direct substitution reaction involving a readily prepared reagent [11].

REACTION PATTERNS OF ACYL PHOSPHATE ESTERS

Methyl acetyl phosphate (MAP) was developed by Kluger and Tsui as an acylating agent for amino groups in cationic regions of proteins [12,13]. The material is an anion, yet it reacts as an electrophile (δ+), an unusual combination. The acyl phosphate methyl ester is very stable in neutral solutions (half life = 160 hours, pH 7, 25 °C) but reacts rapidly with amines.

Detailed studies of the aminolysis reaction of analogous substituted methyl aroyl phosphates give $\beta_{nuc} = 0.9$ (the slope of a plot of log k_{obs} vs. the pK$_a$ of the conjugate acid of the amine nucleophile - a value close to unity indicates a full charge develops in the transition state of the rate determining step) [14]. The expression is:

log k = 0.9*pK$_a$ - 10 (at 25 °C, sec^{-1})

Based on these data, the mechanism for the reaction involves initial reversible addition of the protein amino group to the acyl carbonyl followed by rate-determining expulsion of the phosphate. In the irreversible step, which is rate-determining, there is almost a full positive charge on the amino group since β is near its maximum value. Thus, the reagent reacts in a reversible initial step which leading to greater selectivity than is the case where a reagent is committed upon addition. The choice of methyl esters as opposed to larger alkyl or aryl derivatives is based on a design that minimizes steric effects so that selectivity is controlled by charge density of the protein rather than bulk of the reagent.

Ueno et al. report that MAP reacts with amino groups in the diphospho-glycerate binding site of hemoglobin according to the general reaction pattern proposed by Kluger and Tsui [15-17]. Of the 24 amino groups per $\alpha\beta$ dimer, MAP reacts primarily with two: the α-amino group of β-Val-1 and the ε-amino group of β-Lys-82. There is some reaction with the ε-amino group of β-Lys-144 and with the ε-amino group of α-Lys-99. In his review of chemical approaches to the treatment of sickle cell disease, Manning concludes that MAP is probably the most specific and useful of those chemicals which have been evaluated [18].

The reaction properties of MAP are superficially similar to those of the corresponding 3,5-dibromosalicylates. Both are negatively charged electrophiles. However, in MAP the negative charge is adjacent to the reagent's electrophilic site, while in the dibromosalicylate's the charge is far from the reaction site. nFPLP [8,10] and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) [19] resemble dibromosalicylates in this regard. Also, the acyl phosphate ester leaving group is small and highly polar while the dibromosalicylate leaving group is very large and less polar.

Thus, we expect different absolute reaction patterns and selectivity from the two classes of reagents but similar preferences for reaction near the cationic region where diphosphoglycerate binds. Structural models of deoxy human hemoglobin A reveal that the amino groups of β-Lys-82 are much nearer to the surface of the protein than are the N-terminal valine amino groups (of the β subunits) [20]. Since the dibromosalicylate leaving groups are very bulky, they cannot access the N-terminal valines which are within the channel of the diphosphoglycerate binding site [21].

Based on these results, we proposed that bis(methyl acyl phosphates) would serve as efficient site-directed cross-linkers of hemoglobin. This became the basis for a new technology for efficiently producing red cell substitutes. However, a generalized synthesis needed to be developed. The published literature on acyl phosphate monoesters contained few examples and no tested general methodology.

Dimethyl acetyl phosphate had been prepared by refluxing trimethyl phosphate and acetyl chloride [22]. Difunctional materials cannot be prepared by this since these produce carboxylic anhydrides. Other methods involving substitution reactions had been reported to give impure materials which decompose readily [23].

SYNTHESIS OF ACYL PHOSPHATE ESTERS FOR CROSS-LINKING

We improved the preparation of dimethyl acyl phosphate in stages to permit extension to two-headed materials. First, we found that use of acyl bromides in place of acyl chlorides leads to a much more rapid production of dimethyl acyl phosphates. Then, we found that the sodium salt of dimethyl phosphate will couple with carboxylic acid chlorides at low temperatures in tetrahydrofuran. We did not expect the sodium salt to dissolve, so at first we added crown ethers to promote the reaction. However, we found that the yield improves when the crown ether is omitted, presumably because tetrahydrofuran is capable of solvating the sodium ion. By this procedure we are able to make an acyl dimethyl phosphate from any acid chloride [24].

The use of sodium iodide in acetone to remove one methyl group from a dimethyl acyl phosphate depends on the precipitation of the sodium salt of the phosphate [25]. We did not know if the method extends to two-headed acyl phosphates. With a tetraester, will precipitation occur after one methyl group is removed or will the reaction permit a methyl group from each phosphate diester to be removed? By evaluating the effects of changes of solvent, temperature, and concentration we were able to achieve the desired outcome in a large number of cases.

SITE-DIRECTED MOLECULAR CLAMPS FOR HEMOGLOBIN

We were able to prepare two-headed methyl acyl phosphates where the dicarboxylic acid can be varied. Using these procedures we made a series of these compounds to use as cross-linking reagents of specific bridge length, our "molecular clamps". We made a fumaryl derivative which is an analogue of the Klotz-Walder dibromosalicylate as well as a large set of materials with defined, rigid cross-linking spans.

These materials were reacted with hemoglobin under a variety of conditions, producing a large number of modified hemoglobins which were separated and analyzed structurally and physically. The work has recently been published and the results are discussed in detail [26]. We found that reaction with deoxyhemoglobin produces the most useful species.

CORRELATING OXYGEN AFFINITY AND STRUCTURE

Measurements of the oxygen affinities of these species indicate a systematic relation between oxygen affinity and the length of the bridge between carbonyl groups of the cross-linkers. In FIGURE 1, a plot of log P_{50} vs. cross-link bridge distance is linear for species in which the linkage is between the ε-amino group of β-Lys-82 of one chain and the α amino group of β-Val-1 of the other subunit (designated β'). Thus, oxygen affinity is a simple choice based on the cross-linking reagent's bridging distance. The cross-linked hemoglobins in this case are produced under deoxy conditions.

We also found an opposite correlation slope for β Lys-82 to β' Lys-82 cross-linked species which are generated from oxyhemoglobin (significant amounts of

Log P_{50}, torr
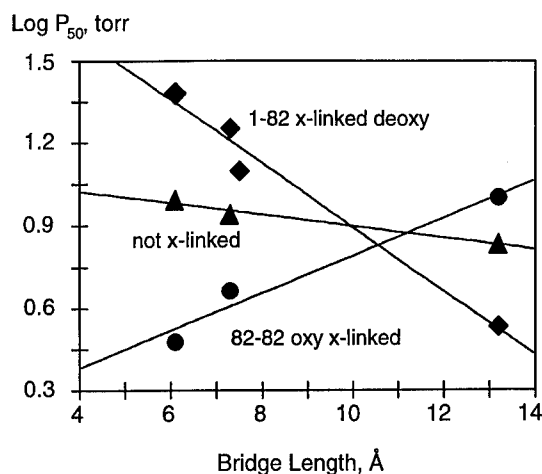
FIGURE 1. Correlation of the logarithm of oxygen affinity value (P_{50}, torr) for a series of cross-linked hemoglobins as a function of the distance of the cross-link span. The 1-82 cross-linked material is produced from deoxyhemoglobin and bis(methyl acyl phosphates) while the 82-82 cross-linked material is produced from oxyhemoglobin and the same reagents.

the 82-82 cross-linked species are generated only under oxy conditions with these reagents). Species which are not cross-linked have no signficant structrue-function dependence. The 1-82' cross-linked species generated under deoxy conditions all have Hill coefficients > 2. Thus, it is clear that the assumption that a residual anion is needed in the diphosphoglycerate site on a cross-linker for low oxygen affinity and a high Hill coefficient is incorrect.

THE PHYSICAL BASIS OF THE OXYGEN AFFINITY CORRELATION

The most significant relationship is the correlation for the 1-82' cross-linked species. What is the physical basis for such a correlation? First, the Hill coefficient of the hemoglobin remains significant after modification. In terms of modern theory, this cooperativity requires that the protein be able to undergo change from the R(relaxed, low energy, oxygenated) to T(tense, high energy, deoxygenated) form, even after modification. Spectroscopic studies in our

laboratory confirm this interpretation [14].   What physical events occur that lead to this changed affinity?

We know that oxygen binds to each of the four hemes of the protein in sequence and that the hemes start out essentially identical. Therefore, binding of oxygen to a heme, which slightly changes the conformation of the heme, must also lead to considerable relaxation of the protein to a lower energy state [27]. In order to release oxygen, the protein must be restored to the higher energy state to reverse the relaxation process. Since the relaxation process involves movement of the protein, the degree to which movement toward a relaxed structure is prevented can be related to the size of the cross-link. A longer cross-linker permits more relaxation and thus causes higher oxygen affinity. By adjusting the length of the cross-link we control the motion of the protein.

DISTANCE OF THE 1-82' CROSS-LINK DETERMINES OXYGEN AFFINITY

The oxygen binding curve of the isophthalyl cross-linked deoxy species appears to be desirable for a useful red cell substitute (P_{50} = 17.8 torr, n = 2.6, 25 °C). However, although the reagent is specific for the diphosphoglycerate site, it produces a heterogeneous collection of modified hemoglobins, corresponding to possible combinations of reaction sites. In addition to the desired Val-1 to Lys-82 cross-linked species with links between Val-1 and Lys-82 of the same subunit, some multiply modified species, some modified on a single β chain and very small amounts linked between Lys-82 of each β subunit.

Since the correlation indicates that the bridge distance is critical in controlling the oxygen affinity, it appeared that it does not matter if there is a different organic structure providing the bridge. Examination of models of the diphosphoglycerate site indicates that the 1-82 cross-link involves a large motion of the α-amino group of a β-Val-1 to bring it into proximity of the ε-amino group of β-Lys-82 of the other subunit. Recent x-ray crystal structures by Dr. Richard Brennan and his co-workers in Portland indicate that such a change does occur. Based on the P_{50} and n_{50} measurements for the modified hemoglobin containing an isophthalyl cross-link between β-Val-1-and β'-Lys-82, we feel that it is likely to provide significant amounts of oxygen in circulation and to be readily oxygenated at atmospheric pressure in the lungs.

Thus, it should be the basis for development of a blood substitute. In order to consider using this as a blood substitute, it must be produced in reasonable quantities.

IMPROVING YIELDS BY GROWING AN EXTRA CLAMP

The yield of 1-82' cross-linked material can be increased in material which already has another cross-link if that material can be induced to change or to add the 1-82' link. The products of the reaction of an amine and a methyl acyl phosphate are an amide and methyl phosphate whose dilution makes the reaction irreversible. So changing the cross-link is not possible. Any "rescue" requires formation of the 1-82' cross-link *in addition* to whatever link might have formed initially. However, once the isophthalyl reagent has formed two amide bonds there is no further reaction possible. A simple solution is to add another functional group that permits further reaction. Thus, a trifunctional reagent which gives the same cross-link distance as the isophthalyl reagent should provide a much higher yield.

With this in mind, we prepared the tris(methyl phosphate) derivative of benzene 1,3,5-tricarboxylic acid (known as trimesic acid). The reagent, trimesyl tris(methyl phosphate) (TMMP) is formed in very high yield from the low-cost commercially available starting material, trimesoyl chloride, and sodium dimethyl phosphate followed by selective demethylation with sodium iodide.

We also used higher temperatures for the modification of hemoglobin with this reagent (60 °C) to increase the rate of the modification reaction. We added the reagent with slow infusion to prevent multiple modification of the same protein molecule. As a result, we produced a much higher yield of material with the critical 1-82' cross-links [1]. We found that there is very little denaturation of hemoglobin during this process and the amount of hethemoglobin formed is less than when the reaction is conducted at room temperature.

Analysis of peptides obtained from a tryptic-Glu-C digest reveals the structures of the major products. These digestive enzymes cleave peptides at sites in which a free amino group is present on a side chain. Acylation of amino groups by our reagents makes the site no longer susceptible to cleavage by these enzymes.
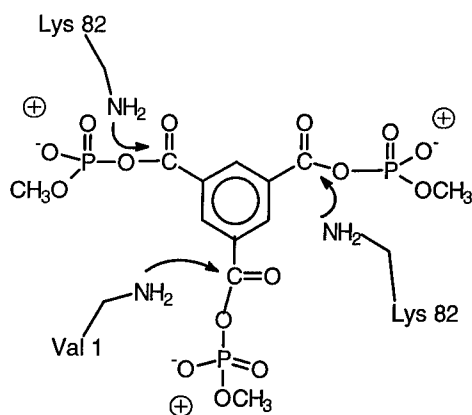
FIGURE 2. Reaction scheme for formation of triply-linked hemoglobin.

As a result, the chromatographic pattern of the resulting peptides differs from that of the native protein. Missing are peptides that would have resulted from cleavage at the now acylated amino groups and new peptides are found corresponding to combination of the uncleaved peptide and cross-linker. Since TMMP introduces an aromatic ring in the cross-linking reaction, the peptide containing the cross-link has a characteristic UV absorbance. The most abundant hemoglobin is triply linked with amide bonds to the ε- amino group of each β-Lys-82 and a third link to the α-amino group of one β-Val-1. A second abundant species has a 1-82' cross-link with the third functional group of the reagent remaining unreacted (the state of the reagent is revealed by ion-spray mass spectral analysis). We also find significant amounts of material with an intrachain cross-link between positions 1 and 82. The HPLC of the tryptic digest of the triply-linked material is shown in FIGURE 3. The trimesylated β chain peptides are indicated as "βT-n" where "n" refers to the normal tryptic peptide.

Since our rationale for the use of the triple-headed reagent suggested all three groups would react, we were puzzled as to why we saw other species. Analysis of the kinetics of the production of the observed species shows they are formed in parallel. Our rationale for the result is based on analysis of structural models. The reagent forms amide bonds and these can be considered as peptide-like extensions
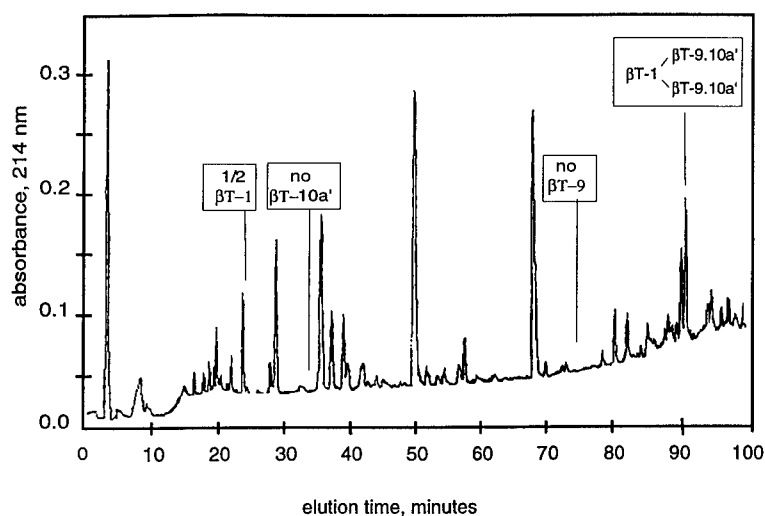
FIGURE 3. HPLC analysis of products of tryptic digest of triply-linked hemoglobin.

to the N-terminus, without control of the stereochemistry of the new bond, which can be cis or trans. Examination of models suggests that one isomer of a trimesyl 1-82 or 1-82' cross-linked species can form a third amide bond while the other isomer cannot.

FURTHER WORK

The acyl phosphate esters have permitted us wide latitude in the design of reagents for the modification of hemoglobin. Our work continues in several directions from this basis. First, we are making more types of acyl phosphate esters to permit more paths for cross-linking and possibilities for rational design of higher species. We are also developing new classes of related materials to permit differentiation of reaction patterns even further. The use of design principles in producing enzyme inhibitors is highly developed and those principles readily extend to the modification of hemoglobin. We are also relating structure to oxygen delivery capabilities and toxicity. We are using new methods, such as ionspray mass spectrometry to obtain detailed structural information.

The use of any of these of materials as a red cell substitute for a clinical application requires a scaled-up procedure, animal and clinical testing. Dr. Diana Pliura and her co-workers at Hemosol, Inc., have been able to modify our methods and have produced the larger amounts of material necessary for biological studies. We have also begun a collaboration with Dr. Andrew Baines at the University of Toronto who is investigating the effectiveness of our materials in oxygen delivery in animal models. Those results will appear in future publications.

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GENETIC ENGINEERING OF MYOGLOBIN AS A SIMPLE PROTOTYPE FOR HEMOGLOBIN-BASED BLOOD SUBSTITUTES.

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ABSTRACT

Site-directed mutagenesis has been used to examine the structural and functional roles of distal pocket residues in regulating O_2 affinity, CO binding, rates of association and dissociation, autooxidation, and hemin loss in mammalian myoglobins and human hemoglobin. In myoglobin, His-E7 inhibits CO binding by requiring displacement of distal pocket water. In the case of O_2 binding, this displacement is compensated by a strong hydrogen bond between the bound ligand and the imidazole side chain. The isopropyl side chain Val-E11 also sterically restricts CO binding. The rates of ligand binding are regulated by distal pocket water displacement, steric restrictions near the iron atom, and an outer more global protein barrier. Autooxidation occurs by two mechanisms, direct dissociation of HO_2 and bimolecular reaction of external O_2 with unliganded heme. Both processes are inhibited markedly by hydrogen bonding interactions with His-E7. Double mutants have been constructed to decrease oxygen affinity, but still prevent oxidation. The apoprotein of His-E7→Tyr myoglobin has been used to extract hemin from other myoglobins and hemoglobin, causing a brown to green color change. This assay has been used to show that polar interactions between residues CD3, E7, E10, F7, and the porphyrin propionates inhibit hemin dissociation markedly.

INTRODUCTION

Assuming blood retention problems can be resolved by chemical or genetic cross-linking, the next step in the design of heme-protein based blood substitutes is to optimize oxygen transport capacity and stability. Five key properties are: (1) relatively poor O_2 affinity (high P_{50}) but high cooperativity;

429

(2) discrimination against CO binding (low M-value); (3) large association and dissociation rate constants; (4) resistance to autooxidation; and (5) low rates of hemin dissociation. In the case of oxygen affinity, two basic strategies can be applied. The first and most popular approach has been to increase P_{50} by enhancing the T-state or low affinity character of the hemoglobin tetramer through either chemical modification [1] or site-directed mutagenesis [2]. The second is to modify the intrinsic properties of the active site in each subunit, hopefully leaving the allosteric properties unimpaired. However, until the pioneering work of Nagai et al. [3] and Springer et al. [4], there had been no systematic attempts to "engineer" the ligand binding sites in hemoglobin and myoglobin for efficient oxygen transport and maximum stability to iron oxidation and hemin loss. The major problem was a lack of background data on the roles of the highly conserved amino acids in the distal pockets of myoglobin and hemoglobin. To remedy this situation, we have been gathering background data on the effects of amino acid replacements in the region of the heme group in mammalian myoglobins and hemoglobins in collaboration with Kiyoshi Nagai's group at the MRC, Cambridge, U. K. , Stephen G. Sligar's group at the University of Illinois, Urbana-Champaign, Anthony J. Wilkinson's group at the University of York, Clara Fronticelli at the University of Maryland, Masao Ikeda-Saito at Case Western University, and Maurizio Brunori's group at the University of Rome. Our goals at Rice University have been to establish the structural mechanisms that regulate ligand binding, autooxidation, and hemin dissociation and then to use this information to design more stable heme proteins with optimal oxygen transport properties.

The complexity of assigning R and T-state rate parameters to the α and β subunits within recombinant tetramers argued against using mutagenesis in hemoglobin to explore the mechanisms of ligand binding and autooxidation [5,6]. Instead, mammalian myoglobin was chosen as a simple, prototypic model for detailed structural, functional, and theoretical analyses. In collaboration with the groups listed above, we have examined the ligand binding properties of 101 single, double, and triple mutants in sperm whale, pig, and human myoglobin, 50 of which were constructed and purified in our laboratory at Rice. The heme pockets of oxy- and deoxymyoglobin are shown in Figs. 1 and 2, respectively. The key amino acids are labeled according to their helical position in the myoglobin folding motif (*i.e.* B10 is position 29 in sperm whale myoglobin, CD3
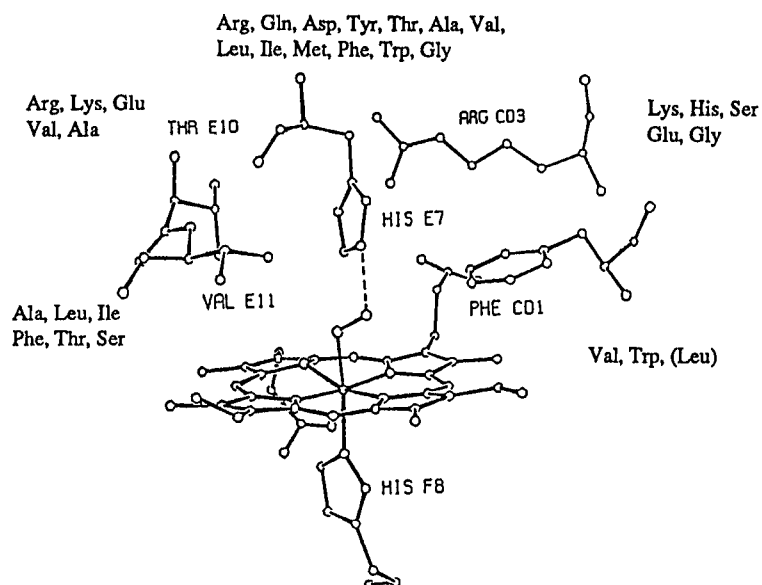
FIGURE 1. View of MbO_2 from the back of the heme pocket looking toward the solvent interface which is behind residues E10 and CD3 [7]. The dashed line indicates hydrogen bonding between bound oxygen and His^{64}(E7).
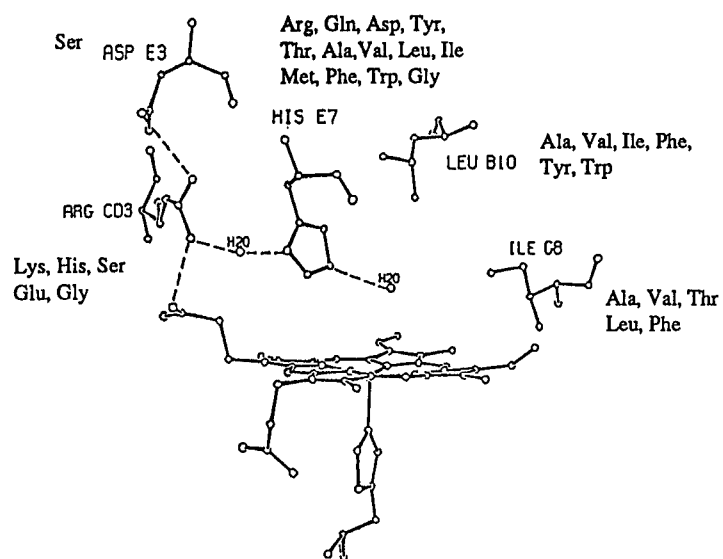
FIGURE 2. View of deoxymyoglobin from the side of the heme pocket (from Val-E11 to Phe-CD1 in Fig. 1) [7]. The dashed lines indicate polar and hydrogen bonding interactions that extend from the solvent interface(left) into the distal pocket.

is 45, E7 is 64 , E11 is 68, *etc.*). Complete lists of amino acid replacements are given beside the native residues. X-ray crystallographic structures of many of these mutants have been determined by George N. Phillips, Jr.'s group at Rice; ns and ps laser photolysis studies have been carried out with Quentin H. Gibson's group at Cornell University; and some of this work has been interpreted by molecular dynamics simulations in collaboration with Ron Elber's group at The Hebrew University. Only summaries of our work are presented in the following sections due to page limitation. Major contributions have been made by other groups and are given in the primary references.

RESULTS

Oxygen affinity, discrimination against CO binding, and kinetics - Since the publication of the first high resolution structures of myoglobin and hemoglobin, the distal histidine has been postulated to play a key role in ligand binding. Mutagenesis studies have quantitated these ideas and provided detailed structural interpretations. As shown in Fig. 1, there is a hydrogen bond between Nϵ of His-E7 and the second bound oxygen atom in MbO$_2$ [7]. In mammalian myoglobins, replacement of His-E7 with Gly, Ala, Val, Ile, Leu, and Phe results in 10 to 100-fold decreases in oxygen affinity suggesting that this interaction contributes -1 to -2 kcal/mol to the binding free energy in the native protein (Table I, second column and [4]). The His-E7$\rightarrow$Gln mutation is the most conservative in terms of oxygen affinity, presumably because the amide nitrogen of the side chain can still hydrogen bond with bound O$_2$; however, even in this case, there is a 6-fold decrease in affinity.

His-E7 is also largely responsible for discrimination against CO binding in myoglobin, as judged by the large increases in M (K$_{CO}$/K$_{O2}$) produced by the various E7 mutations listed in Table I. The primary cause of this discrimination is the presence of a distal pocket water molecule that is hydrogen bonded to His-E7 in deoxymyoglobin but not coordinated to the iron atom (Fig. 2, [4,7,9]). CO must displace this water before binding to the iron atom, and the free energy required to break the H$_2$O-His hydrogen bond decreases the equilibrium association constant. In the case of O$_2$ binding, this unfavorable effect is compensated by the formation of a stronger hydrogen bond between His-E7 and the polar Fe(II)-O-O complex. The results for the Val-E11 substitutions show that this residue also restricts CO binding. Decreasing the size of the E11 residue

TABLE I. Comparison of Oxygen Equilibrium Association Constants (K_{O2}) and CO/O_2 Partition Constants (M) for Sperm Whale Myoglobin and R-State α and β Subunits of Human Hemoglobin at 20°C, pH 7.0.

| Mutation | K_{O2} | | | M | | |
	Mb^a (μM^{-1})	α^b (μM^{-1})	β^b (μM^{-1})	Mb	α	β
Native	1.2	2.3 (3.0)^c	4.5 (3.0)^c	23	273	220
H(E7)G	0.088	0.4	2.7	1,700	7,000	140
H(E7)Q	0.18	0.8	2.9	460	1,900	240
H(E7)F	0.0074		2.0	11,000		210
V(E11)A	1.2	3.1	6.7	47	2,700	87
V(E11)L	3.4	6.0	6.0	14	140	100
V(E11)I	0.22	2.4	0.43	9.1	83	58

^{a} Data for sperm whale myoglobin taken from references [4,8].

^{b} Data for human hemoglobin taken from reference [5].

^{c} The affinity constants for R-state subunits have an error of $\pm$ ~40% and a single equal value of 3.0 μM^{-1} has been suggested by several authors [5,6,12].

to Ala causes M to increase roughly 2-fold and increasing its size to Ile causes M to decrease by the same amount. However, the key cause for discrimination against CO is clearly the polarity of residue E7.

The kinetics of ligand binding are more difficult to interpret. At least three steps are involved in association: (1) entry into the protein and movement into the distal pocket defined by residues B10, B13, CD1, and G8 (Fig. 2); (2) movement past residues B10 and E11 and up to the iron atom; and (3) bond formation . The rates of the innermost process are governed by the size of residues B10 and E11 and the presence or absence of water hydrogen bonded to residue E7 [10,11]. The pathway for ligand entry into the protein remains controversial and has not been defined experimentally [10].

Applications to hemoglobin engineering -   As shown in Table I, E7 and E11 substitutions produce similar effects in Mb and R-state α subunits, although the changes in the latter protein are generally smaller. These results point to a key

role for His-E7 in regulating O_2 and CO binding in α subunits. In contrast, none of the mutations except, Val-E11→Ile produce much effect on R-state β subunits. Thus, the R-state β heme pocket appears to be plastic and can accommodate substantial changes without alteration in functional properties [see also 12]. The distal pocket of R-state β subunits also appears to be more apolar than that in either α subunits or Mb, and there does not appear to be a hydrogen bond between bound O_2 and β His-E7. However, there must be some compensating favorable interactions since the affinities of the two hemoglobin subunits appear to be equal within a factor of 2. In contrast, ligand binding to T-state β subunits is greatly affected by E7 and E11 mutations [6], and these results point out the need to characterize the active sites of the subunits in both quaternary conformations.

Autooxidation - In native myoglobin and most mutants containing His-E7, autooxidation occurs by two mechanisms which are described in Fig. 3 and discussed in detail by Brantley et al. [13,14]. At high oxygen concentration, the predominant pathway is direct dissociation of neutral superoxide from the protonated Fe-O-O-H$^+$ complex (intermediate A). At low oxygen concentrations, autooxidation occurs by a bimolecular reaction between external O_2 and deoxymyoglobin containing weakly coordinated water molecule. The neutral side chain of His-E7 inhibits both processes by hydrogen bonding to the bound oxygen (intermediate B). Replacement of His-E7 with apolar side chains increases the rate of autooxidation 1,000-fold and causes the superoxide dissociation mechanism to predominate. Increases in autooxidation are also observed when the polarity of the distal pocket is increased (Val-E11→Thr, Ser mutations), the net anionic charge near the edge of the porphyrin is increased (Thr-E10→Glu; Arg-CD3→Glu), and when the volume of the distal pocket is increased (Val-E11→Ala; Leu-B10→Ala, Val) [14].

The dominant mechanism for inhibiting autooxidation is hydrogen bonding between the bound oxygen and the neutral imidazole side chain. This interaction prevents net protonation of Fe-O-O since the pK_a for formation of the imidazole anion is extremely large. This mechanism suggests that there should be a direct correlation between K_d (P_{50}) and the rate of autooxidation. Experimental verification of this relationship is shown in Fig. 4. This correlation presents a serious dilemma for engineering hemoglobin as an extracellular blood substitute. One of the major goals is to increase P_{50} for more efficient transport, but as
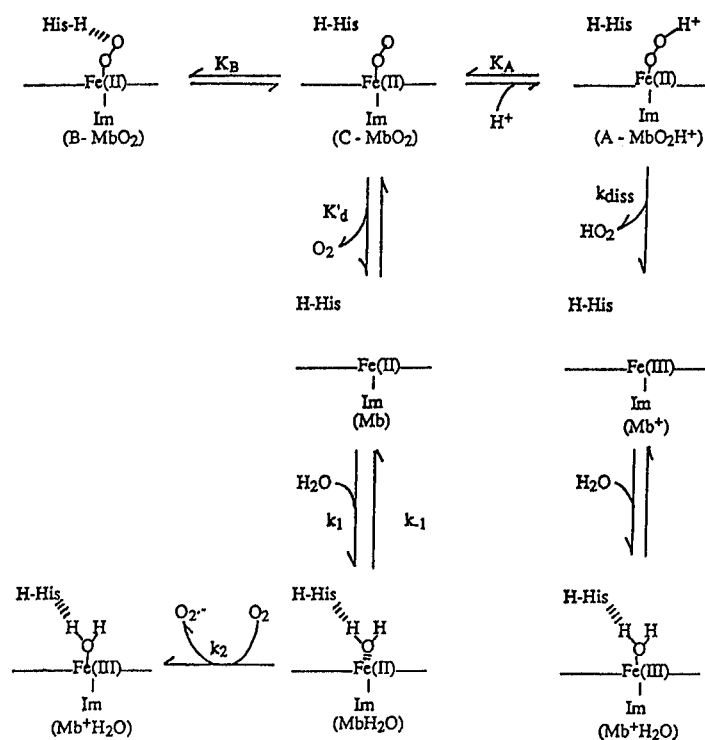
FIGURE 3. Proposed Chemical Mechanism for the Autooxidation of Myoglobin

shown in Fig. 4, a decrease in oxygen affinity almost invariably leads to an increase in the rate of autooxidation.

Our initial attempts to examine and alleviate this problem are shown in Table II. Carver et al. [15] discovered that replacing Leu-B10 with Phe produces a protein which is remarkably stable to autooxidation ($t_{1/2}$ ~6 days vs. 12 hours for wild-type or native Mb). However, this mutant also has an extremely high affinity for O_2 which would be detrimental for both oxygen transport and storage. Part of the reduction in autooxidation rate appears to be due to favorable electrostatic interactions between the phenyl multipole and bound oxygen, and part is due to exclusion of water from the distal pocket. We have constructed double mutants in an effort to take advantage of the role of Phe-B10 in inhibiting
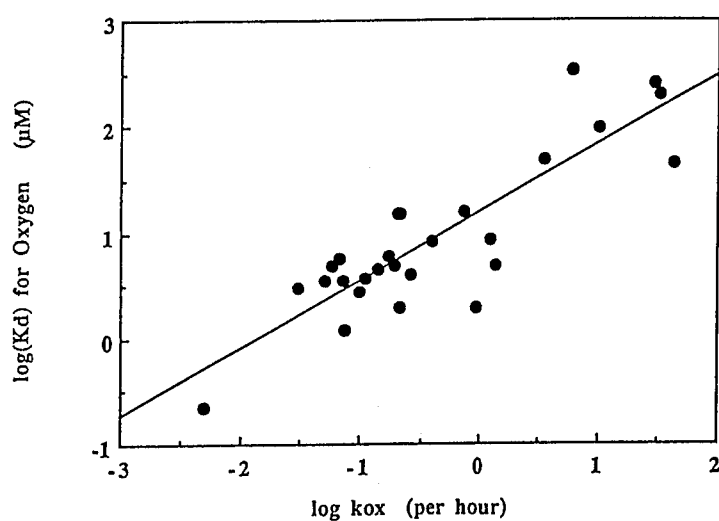
FIGURE 4. Plot of the log of the equilibrium oxygen dissociation constant versus the log of the rate constant for autooxidation in air at 37°C, pH 7.0 in presence of EDTA, catalase, and superoxide dismutase [see 14].

TABLE II. Comparison of Oxygen Equilibrium Dissociation Constants (K_d or P_{50}) and Autooxidation Rates (k_{ox}) in Air at 37°C, pH 7.0 for Single and Double Mutants of Sperm Whale Myoglobin. Data were taken from references [13,14]. In sperm whale myoglobin, positions 29, 64, and 68 correspond to helical positions B10, E7, and E11, respectively (see FIGURES 1 and 2).

Single Mutants			Double Mutants		
Replacement	K_d μM	k_{ox} h^{-1}	Replacements	K_d μM	k_{ox} h^{-1}
Native	3.3	0.060	Native	3.3	0.060
L29F	0.22	0.005			
H64Q	15.	0.21	L29F-H64Q	5.3	0.072
V68I	16.	0.75	L29F-V68I	1.2	0.075
V68F	5.9	0.069	H64Q-V68F	15.	0.20

autooxidation and, at the same time, to reduce oxygen affinity by weakening hydrogen bonding with the E7 residue (His-E7→Gln) or sterically hindering the bound ligand (Val-E11→Ile). The results for the Leu-B10,Gln-E7 mutant are encouraging but clearly not good enough (Table II). Interestingly, the single mutant Val-E11→Phe gives similar results, a 70-100% increase in K_d with little increase in k_{ox}. The latter effect is presumably also due to limited solvent accessibility. The double mutant Gln-E7, Phe-E11 causes a 5-fold increase in K_d, but there is a 3-fold increase in k_{ox} and the M value is high (data not shown). This work is being extended to other double and triple mutants. It appears likely that the optimum protein will be a result of mutating both "first shell" amino acids which are in direct contact with the bound ligand and "second shell" residues which influence the exact conformation of the first shell side chains.

Hemin dissociation - In order to develop a rapid and convenient assay for hemin dissociation, we replaced His-E7 with Tyr in sperm whale myoglobin to produce a protein with a distinct green color. Egeberg et al. [16] had made this mutant previously and suggested that its abnormal spectrum is due to coordination between the phenol side chain and the oxidized iron atom. George Phillips' group has confirmed this directly by determining the structure of the His-E7→Tyr (H64Y) mutant of sperm whale metmyoglobin [17]. When excess apoprotein of this mutant is mixed with either native ferric myoglobin or hemoglobin, the solutions turn from brown to green, and the time course of hemin dissociation from the holoprotein can be measured either in the visible wavelength region at 600 nm or in the Soret region at 410 nm (Fig. 5A).

The single mutant H64Y has a relatively weak affinity for hemin compared to native protein, and the apoprotein is thermally less stable. In order to increase both hemin affinity and stability, the double mutant His-E7→Tyr, Val-E11→Phe (H64Y-V68F) was constructed. As shown in Fig. 5B, the rate of hemin dissociation from the double mutant is 3 times smaller than that from the single H64Y mutant and only 2-4 times greater than that for native myoglobin under comparable conditions (Fig. 6A). In addition the double mutant apoprotein shows much less denaturation during prolonged incubation at 20°C [17]. However, both mutant apoproteins denature at 37°C. In order to carry out experiments at elevated temperatures, the reactions have to be carried out in 1M sucrose. Otherwise the assays have to be carried out at lower pH values in order to speed up the hemin dissociation reaction.
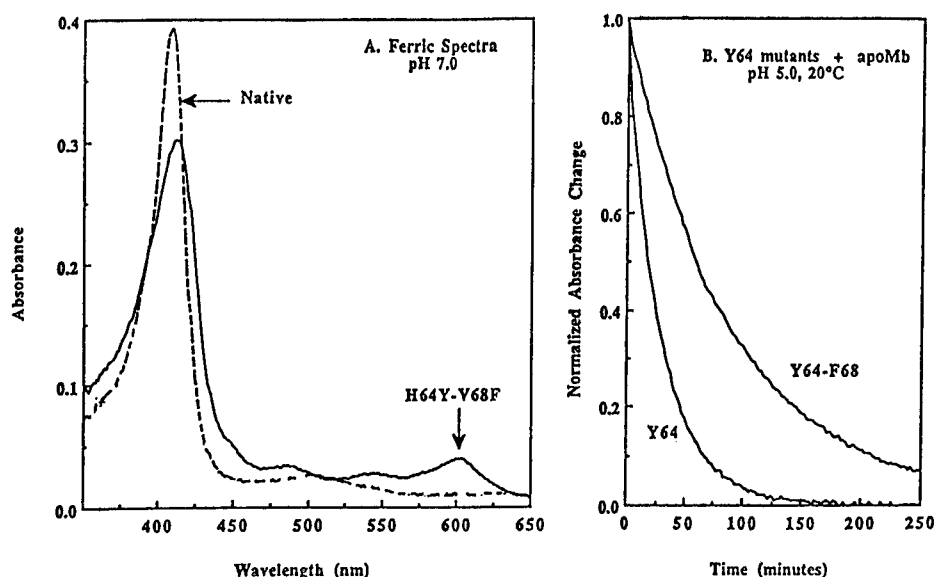
FIGURE 5. Properties of H64Y mutants of sperm whale myoglobin. *A.* Absorption spectra of ferric native and H64Y-V68F myoglobin at pH 7.0. *B.* Time course for hemin dissociation from H64Y and H64Y-V68F Mb. The mutants were mixed with a 10-fold excess of native apomyoglobin at pH 5.0, 20°C. Absorbance changes were followed at 410 nm and the observed rate constants were 2.0 and 0.66 h^{-1} for the single and double mutants, respectively.

Comparisons between several myoglobin mutants are shown in Fig. 6. These results show clearly that polar interactions between the porphyrin propionates and residues Ser-F7(92), His-E7(64), Arg-CD3(45) play a key role in inhibiting hemin dissociation (see Fig 2). The pH dependence indicates that protonation of the propionates and/or the distal histidine facilitates hemin loss. Similar experiments with native human hemoglobin, $(\alpha^+)_2(\beta CO)_2$, and $(\alpha CO)_2(\beta^+)_2$ hybrids have been carried out with Dr. Antony J. Mathews from Somatogen. The rates of hemin loss from the α and β subunits are, respectively: 1.4 and 13 h^{-1} at pH 5.0, 20°C and 0.4 and 7.2 h^{-1} at pH 7.0, 37°C, 1 M sucrose. The comparable rates for native sperm whale myoglobin are 0.32 h^{-1} at pH 5.0, 20°C and 0.04 h^{-1} at pH 7.0, 37°C, 1 M sucrose (Fig. 6). These results confirm and quantitate the earlier conclusions of Bunn and Jandel [18] and Benesch and
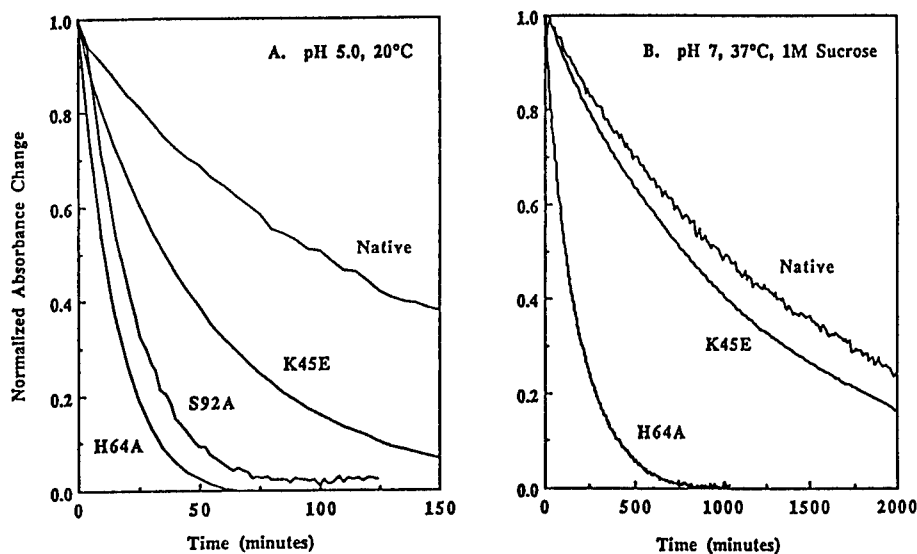
FIGURE 6. Time course for hemin dissociation from myoglobins at _A._ pH 5.0, 20°C and _B_, pH 7.0, 37°C, 1 M sucrose. In each reaction, ~5μM myoglobin was mixed with 50-100 μM H64Y-V68F apoprotein and the reaction followed at ~410 nm. The observed rates at pH 5 for native, K45E, S92A, and H64A myoglobins were 0.32, 1.1, 3.3, and 4.0 h^{-1}, respectively. The rates at pH 7 for native, K45E, and H64A were 0.040, 0.054, and 0.34 h^{-1}, respectively.

Kwong [19] using isotope exchange techniques and human serum albumin to extract the hemin. The β subunits lose hemin 10 times more rapidly than α subunits, and myoglobin shows a further 10-fold reduction in rate under comparable conditions. These differences correlate inversely with the basicity of the residue at CD3: Ser for β subunits, His for α subunits, and Arg for Mb.

The H64Y-V68F double mutant is being engineered to improve its thermal stability even further in order to provide a more reliable assay under physiological conditions. Clearly, much more work is needed to screen the current library of myoglobin mutants, to examine the effects of pH, temperature, and solvent additives, and to apply the assay to hemoglobin blood substitutes. Then systematic engineering of more stable proteins can be attempted.

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A COMPARISON OF RATES OF HEME EXCHANGE: SITE-SPECIFICALLY CROSS-LINKED *VERSUS* POLYMERIZED HUMAN HEMOGLOBINS

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ABSTRACT

The stability of the heme-globin interaction of chemically modified human hemoglobin (Hb) was tested by measuring rates of heme loss from methemoglobin. Heme transfer from methemoglobin to human serum albumin was measured by rapid-scanning spectrophotometry, and the resulting absorption matrices were analyzed by singular value decomposition. Unmodified human HbA_0, hemoglobin cross-linked between β subunits with either 2-nor-2-formylpyridoxal 5'-phosphate or 3,5-(dibromosalicyl)fumarate (DBBF), hemoglobin cross-linked between α subunits with DBBF, and pyridoxalated hemoglobin polymerized with either glycolaldehyde or glutaraldehyde were tested. Initial rates were evaluated by fitting the time courses to a biexponential equation using a matrix least squares curve-fitting algorithm. Reaction rates fell into two classes: (1) HbA_0 and the site-specifically cross-linked hemoglobins, with biphasic rates of heme loss of 0.02 and 0.004 min^{-1}, and (2) polymerized hemoglobins, with 10-20-fold higher rates at 0.5 and 0.03 min^{-1}. The total fitted amplitudes of the reaction depended upon the specific modification: $\beta\beta$-cross-linked Hbs < $\alpha\alpha$-cross-linked Hb $\approx$ glycolaldehyde polymerized Hb < glutaraldehyde polymerized Hb < HbA_0.

INTRODUCTION

Designing hemoglobin-based red blood cell substitutes requires identifying the fundamental properties of oxygen transport by red blood cells and conferring those properties on cell-free solutions of hemoglobin without inflicting untoward side-effects. Not everything about red cell function is known, but a few important properties are obvious. (1) Red blood cells carry hemoglobin at high intracellular concentrations, providing high O_2-carrying capacity and favoring the tetrameric form of hemoglobin over dimer formation. (2) Human red blood cells produce 2,3-diphosphoglycerate (2,3-DPG) during their normal glycolytic metabolism; 2,3-DPG binds to hemoglobin and lowers oxygen affinity, thus increasing the efficacy of oxygen release to tissues. (3) Human red blood cells produce an intracellular methemoglobin reductase enzyme system that minimizes the amount of methemoglobin inside normal red blood cells to $\leq$3-5% of total cellular hemoglobin.

The first two properties have been addressed in the design of hemoglobin-based blood substitutes. Both prevention of hemoglobin tetramer dissociation and alteration of intrinsic oxygen affinity can be achieved by chemical cross-linking or genetic manipulations [for a review, see ref. 1]. However, the third property listed above has not been met. Autooxidation of native, chemically modified or genetically altered hemoglobins has not yet been subject to control by design. And unfortunately, the rate of autooxidation of ferrous (Fe^{2+}) oxyhemoglobin to ferric (Fe^{3+}) methemoglobin has been found so far to be inversely proportional to O_2 affinity [2].

Methemoglobin formation is doubly disadvantageous. (1) It does not bind O_2, and (2) oxidation is the first step in hemoglobin denaturation. Ferric heme has a reduced affinity for globin [3], leading to heme loss and apoprotein precipitation. This may be particularly undesirable in cell-free hemoglobin solutions, because in the absence of the red blood cell membrane barrier, other

oxidatively sensitive membranes, such as endothelial membranes, become exposed directly to free heme.

To address this issue, we measured rates of heme loss from different methemoglobin derivatives as an indication of the effects of protein modification on the stability of the heme-globin linkage.

MATERIALS AND METHODS

All hemoglobin samples were derived from human blood. Three of the hemoglobin samples used in these experiments were provided by the Letterman Army Institute of Research Hemoglobin Production Facility. These included: hemoglobin A_0 (HbA$_0$), hemoglobin cross-linked between α subunits ($\alpha\alpha$Hb) by 3,5-(dibromosalicyl)fumarate (DBBF) [4], and glycolaldehyde polymerized pyridoxalated hemoglobin [5]. The polymerized sample was produced in collaboration with Duncan Pepper and Shirley MacDonald of the Scottish National Blood Transfusion Service. Hemoglobin cross-linked between β subunits with 2-nor-2-formylpyridoxal 5 '-phosphate ($\beta\beta$Hb-NFPLP) was provided by Joa Bakker of the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service [*see ref.* 6]. Hemoglobin cross-linked between β subunits by DBBF ($\beta\beta$Hb-DBBF) [7] and glutaraldehyde polymerized pyridoxalated hemoglobin [8] were provided by Angelo Zegna of the Blood Research Division, Letterman Army Institute of Research.

The heme exchange assay was adapted from the method of Benesch and Kwong [9]. Methemoglobin was prepared by reacting hemoglobin with a 1.2-fold excess of potassium ferricyanide to heme at room temperature in 0.1 M bis-Tris buffer, pH 7.0. Ferro- and ferricyanide were removed by gel filtration on a Sephadex G-25 column equilibrated with 50 mM bis-Tris buffer, pH 7.5, containing 0.1 M Cl$^-$.

The assay conditions were as follows: 50 μM (in heme) methemoglobin and 50 μM human serum albumin (HSA), 0.25 M Tris buffer, pH 9.0, and

20°C. The reaction was initiated by the addition of HSA. A rapid-scanning spectrophotometer (LT Quantum 1200), scanning from 400 to 800 nm in 200 ms, was used to monitor the reaction as heme was lost from methemoglobin and bound to HSA. For each time point, four spectra were collected and averaged into a single spectrum with a time resolution of 0.8 s. Data collection was under computer control to provide exact intervals in time between spectral measurements from 0.5-10 min, depending on how quickly the reaction occurred.

For data analysis, spectra from 480 to 650 nm at 1-nm intervals were combined in a single absorbance matrix (A) with a total of 171 wavelengths, or rows. The number of columns in A were determined by the number of time points in the reaction. Two matrix procedures were used for analysis of A: singular value decomposition (SVD) and matrix least squares. A brief description of these methods is given here. A more rigorous discussion can be found in Vandegriff and Shrager [10].

SVD determines the number, or rank, r, of optical species undergoing a transition in A by decomposing the A matrix into three other matrices,

$$A = USV^T \tag{1}$$

where the columns in U are linear combinations of the independent spectra of each component in A, the columns of V are linear combinations of the transitions of each component in A, and S gives the singular values of A. For columns i where $i > r$, U column i and V column i contribute negligibly to the signal in A, and the minimal form of the data in A can be created from the columns of U, S, and V that contain signal.

The matrix A also is described by two other matrices,

$$A = DF^T \tag{2}$$

where the columns in D contain the spectra that are changing, and the columns in F give the transition curves for the spectra in D. F can be fitted for by predicting a model for the transitions in A and using matrix least squares to

adjust the parameters in **F** by curve fitting to minimize the residual sum of squares for **A** - **DF**^{T}, where **A** is known. To model **F** for these reactions, a biexponential equation was used with equal amplitudes for the two phases, *i.e.*,

$$k_{obs} = 0.5A_T(e^{-k_ft} + e^{-k_st}) \tag{3}$$

where k_{obs} is the rate of methemoglobin disappearance, A_T is total change in amplitude, and k_f and k_s are, respectively, the fast and slow rate constants.

RESULTS

The heme exchange reaction was measured for purified HbA_0, three site-specifically cross-linked human hemoglobin tetramers, and two polymerized pyridoxalated human hemoglobins. The reaction of DBBF with oxyhemoglobin cross-links the tetramer primarily between βLys(82) residues in the β pocket [7], with a smaller percentage cross-linked between $β_1$Lys(82) and, most likely, $β_2$Lys(144) [11]. NFPLP cross-links deoxyhemoglobin between the N-terminal amino group of one β subunit [*i.e.*, $β_1$Val(1)] and $β_2$Lys(82) of the opposite β subunit [12]. The reaction of deoxyhemoglobin with DBBF cross-links the tetramer between αLys(99) residues in the central cavity of the hemoglobin molecule [13]. The samples of polymerized pyridoxalated hemoglobin used in this study were prepared with the multifunctional aldehyde, glutaraldehyde (polyGlutar) or with the monoaldehyde, glycolaldehyde (polyGlycol).

The spectral change during heme exchange from $metHbA_0$ to HSA is shown in **Figure 1A**. As can be seen from the experimental spectra compared with the spectrum of methemalbumin (dashed line, **Figure 1A**), the reaction did not go to completion. Under these conditions, when apohemoglobin becomes ~50% of the total, protein denaturation begins to occur, and the spectral baseline shifts.

The three-dimensional absorbance matrix **A** for the experiment with HbA_0 is shown in **Figure 1B**. Wavelength is shown along the x-axis from 480
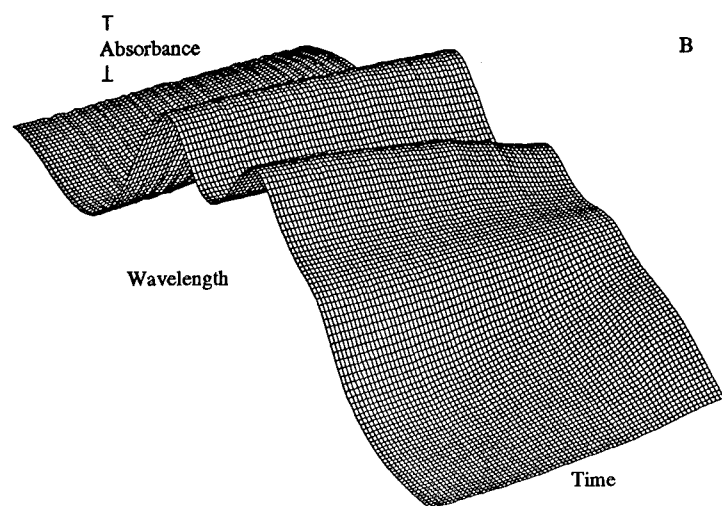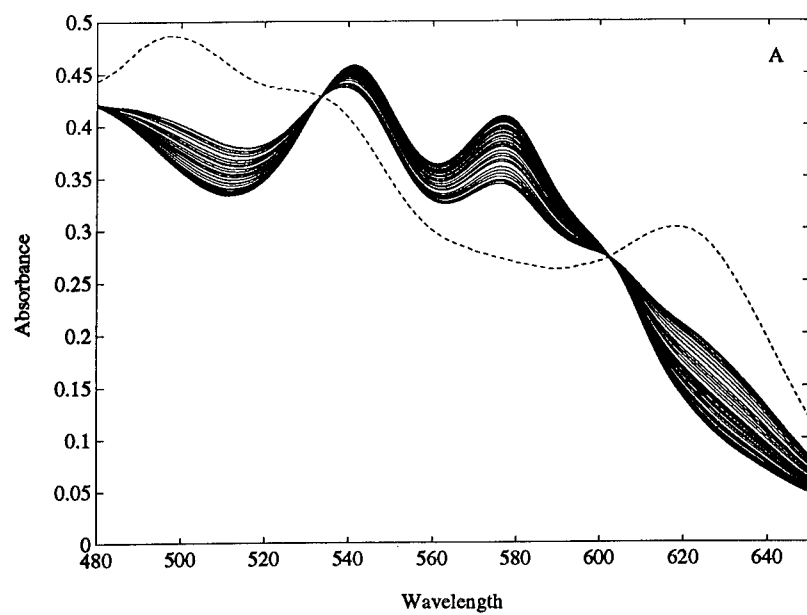
FIGURE 1. (A) *Solid lines*, absorption spectra during heme exchange from metHbA$_0$ to HSA. *Dashed line*, methemalbumin. (B) Matrix A.

to 650 nm, absorbance is presented along the y-axis, and time is represented by the z-axis. The **S** matrix from SVD of **A** was used to estimate the rank of **A** by plotting
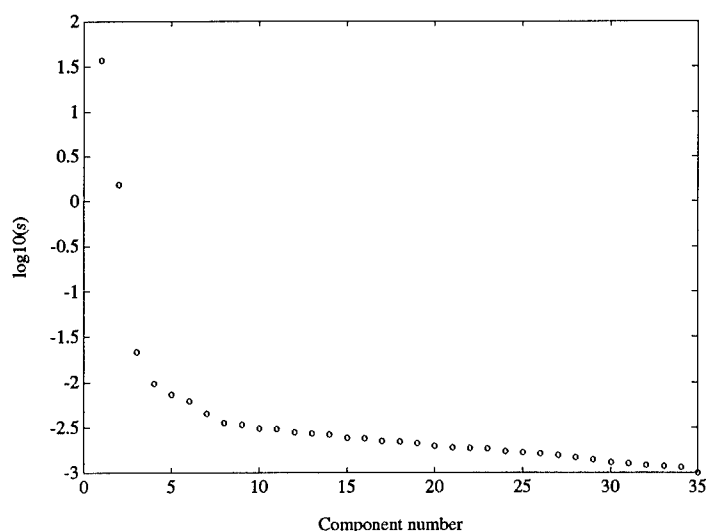
FIGURE 2. Evaluation of the rank of A.

$\log_{10}(s_{i,i})$ *versus* i (**Figure 2**). The number of components that stand out above the declining smooth curve provides an estimate of rank. It is difficult to determine rank exactly, but it is clear that beyond the 3rd-5th component, a signal is difficult to resolve above random noise. This was found to be the case for all of the hemoglobin samples. As a result, minimal matrices **A** were constructed from each of the hemoglobin data sets from the top five components of SVD matrices **U**, **S**, and **V**.

A matrix least squares algorithm was used to fit for the amplitudes and rates of the reactions. The non-polymerized tetrameric hemoglobin samples could be fit to either mono- or biexponential expressions, but the residual sum of squares were ~2-fold less using the biexponential function in Eq. (3). The polymerized hemoglobin time courses could not be fit to a single exponential function.

The amplitudes of the two phases in the biexponential expression were either constrained to 50% of the total amplitude [as in Eq. (3)] or allowed to float. In either case, the final best-fit amplitudes for the two kinetic phases for all of the modified hemoglobins were ~50% of the total. For HbA_0, the fractional amplitudes converged to 30% of the total for the fast phase and 70% for the slow phase, but this fit was not improved over the fit to Eq. (3). Thus, Eq. (3) was used for all of the final curve-fitting analyses.

Matrix least squares was performed by setting F = Eq. (3) and allowing the parameters A_T, k_f, and k_s to be adjusted to minimize the residuals. The columns in F represent the time courses for the disappearance of methemoglobin and the appearance of methemalbumin. The best-fit parameters for these six time courses are given in **Table I**. The corresponding time courses for the disappearance of methemoglobin are presented in **Figure 3**.

Initial rates of the reaction for these six hemoglobin samples fall into two distinct classes: (**Class I**) unmodified or cross-linked tetrameric human hemoglobin and (**Class II**) polymerized human hemoglobin. The initial rates for the reactions within a class are the same, with variations only in total amplitude. **Class I** hemoglobins show fast and slow rates of 0.02-0.03 and 0.004-0.005 min^{-1}, respectively. **Class II** hemoglobins show respective rates at least 10-fold greater, *i.e.*, 0.4-0.5 and 0.03-0.04 min^{-1}. The total fitted amplitudes for the reactions were for **Class I**: $\beta\beta Hb < \alpha\alpha Hb < HbA_0$, and for **Class II**: glycolaldehyde polymerized Hb < glutaraldehyde polymerized Hb.

DISCUSSION

The success of modified hemoglobin solutions as red cell substitutes will depend on the efficacy of oxygen transport balanced against toxic effects. Some of the toxic reactions of cell-free hemoglobin solutions have been resolved. Renal injury can be alleviated by preventing dimer formation and

TABLE I

INITIAL RATES OF HEME EXCHANGE[a]

Hb	Amplitude (total)	k_f (min^{-1})	k_s (min^{-1})
CLASS I			
$\beta\beta$ (DBBF)	0.16	0.02	0.004
$\beta\beta$ (NFPLP)	0.18	0.03	0.005
$\alpha\alpha$ (DBBF)	0.39	0.02	0.004
A_0	0.67	0.02	0.004
CLASS II			
polyGlycol	0.38	0.42	0.031
polyGlutar	0.49	0.49	0.035

[a]Conditions: [metHb] = 50 μM (in heme); [HSA] = 50 μM; Tris buffer, pH 9.0; 20°C.

excretion. Pyrogenic effects of red cell stromal phospholipids and/or endotoxin contamination can be prevented by following rigorous purification strategies. The causes and prevention of other documented toxicities are still being debated [*for reviews, see refs*. 1 and 14]. Hypertension and free radical damage are two key areas under study. Free radicals are of primary concern because oxygen-free radicals are formed during hemoglobin oxidation. In the absence of methods to prevent autooxidation, oxidative by-products must be dealt with in the overall design of hemoglobin-based blood substitutes.

Hemoglobin subunits have unequal affinities for heme. β subunits lose their hemes ~10-fold faster than α subunits [3]. Therefore, with unmodified HbA$_0$ and site-specifically cross-linked hemoglobin tetramers, heme loss from β globin likely provided the majority of the observed absorption change. Release
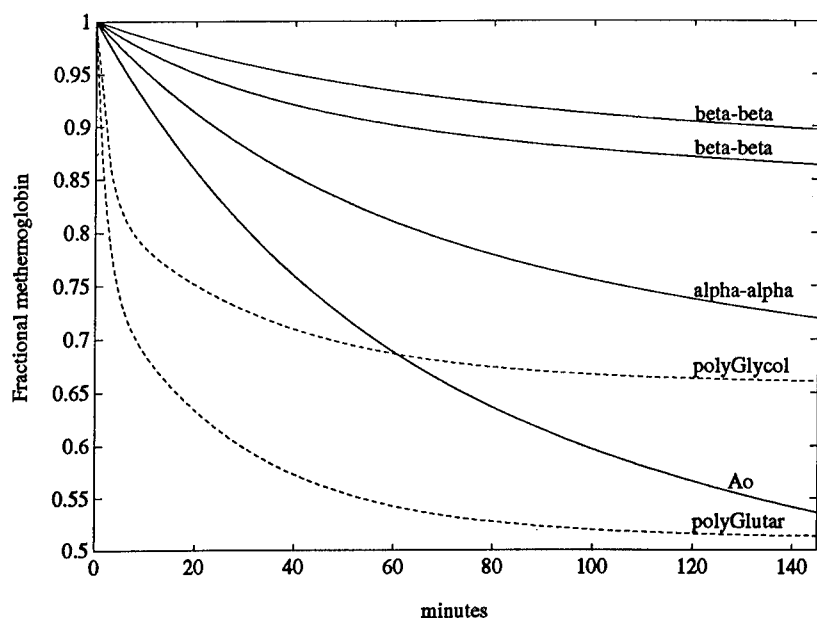
FIGURE 3. Matrix least squares best-fit curves for the disappearance of methemoglobin: F column 1. Class I, shown in *solid lines* from top to bottom: $\beta\beta$-cross-linked Hb (DBBF), $\beta\beta$-cross-linked Hb (NFPLP), $\alpha\alpha$-cross-linked Hb (DBBF), and HbA_0. Class II, shown in *dashed lines* from top to bottom: glycolaldehyde polymerized pyridoxalated Hb (polyGlycol) and glutaraldehyde polymerized pyridoxalated Hb (polyGlutar).

of heme from α subunits is more difficult to measure because of globin precipitation [9, 15]. The smaller rate found in these experiments may correspond to heme release from α subunits, but the overall extent of the reaction does not allow a truly definitive interpretation at this time.

Our results suggest that site-specifically cross-linking hemoglobin does not alter the heme-globin interaction in a way that affects rates of heme loss. In contrast, polymerization causes a marked increase in rates of heme loss. This is consistent with a Mössbauer spectroscopic study of hemoglobin that

showed that the heme pocket is opened up by polymerization [16]. As a result, polymerized hemoglobins may be more susceptible to oxidation and heme loss.

The fast rate for the polymerized hemoglobins is 10-20-times faster than the fast rate for the non-polymerized hemoglobins (0.4 *versus* 0.02-0.03 min^{-1}), and the slow rate for the polymerized samples is equal to the fast rate for the non-polymerized samples. Thus, two explanations are possible: (1) the two rates for polymerized hemoglobin may correspond to heme loss by both subunits, with both rates being markedly faster than the corresponding rates for non-polymerized samples, or (2) the fast phase may correspond to destabilized heme linkages in 50% of the polymerized monomers, while the remaining slower 50% of the reaction corresponds to a normal rate of β-subunit heme loss as in the non-polymerized hemoglobins. Because each kinetic phase contributes equally to the total amplitude of the reaction, the first explanation, *i.e.*, that the two rates reflect α- and β-subunit heme loss, is more probable.

The different amplitudes for the reactions within each class suggest that these modifications affect the probability of the reactions more than the initial rates. HbA$_0$ gave the largest amplitude. As suggested by Benesch and Kwong [9], this is likely because HbA$_0$ dissociates into dimers, which are less stable, whereas, cross-linked hemoglobins do not dissociate. The different amplitudes observed for the polymerized hemoglobins may be the result of the polymerizing agents. Glycolaldehyde is a monoaldehyde that is shorter and not as reactive as the multifunctional glutaraldehyde; the glycolaldehyde reaction is more controlled and forms fewer intermolecular cross-links [5].

Our assay conditions do not reflect *in vivo* pH or temperature but are necessary for the spectral measurements and apoprotein stability. A new method, using a mutant apomyoglobin that becomes green upon heme binding, is being developed (*see*, J.S. Olson's chapter, this volume). Methods to improve the stability of the apoproteins so that the reaction can be followed to

completion at physiological pH and temperature will provide more definitive analyses of the reaction and possibly better extrapolation to clinical situations.

Authors' note: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the U.S. Department of the Army or the Department of Defense.

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INITIAL CLINICAL EXPERIENCE WITH A RATIONALLY DESIGNED, GENETICALLY ENGINEERED RECOMBINANT HUMAN HEMOGLOBIN

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The need to develop a blood substitute is now urgent because of the increasing concern over blood-transmitted viral and bacterial pathogens[1]. During the past 50 years, there have been a number of attempts to develop a hemoglobin-based red blood cell substitute. However, developing a hemoglobin-based oxygen carrier (HBOC) as a blood substitute presents several key challenges. Hemoglobin, as a cell - free protein, cannot be used without modification because (1) the oxygen affinity of the cell-free molecule, in the absence of 2,3 - disphosphoglycerate, is too high to allow adequate unloading of oxygen to the tissues[2], and (2) the molecule, as a tetramer, dissociates into $\alpha\beta$ dimers[3]. This dissociation into $\alpha\beta$ dimers leads to a short plasma half - life, due to rapid renal filtration[4-6], and can result in kidney damage[3-5].

Another major challenge in the successful development of a HBOC is the sourcing of hemoglobin. If human or animal red cells are used as the source, the protein must be chemically modified, and the potential to transmit infectious disease agents still exists. One also has a limited donor pool in the case of human hemoglobin derived HBOCs.

457

SHOEMAKER ET AL.

Somatogen's approach to the development of a HBOC combines the
utility of protein engineering with the advantages of producing the
molecule in microorganisms. This allows the facile production and
testing of new hemoglobins, that are optimized for stability, oxygen
delivery and plasma half-life. In addition, the ability to produce
human hemoglobin by fermentation eliminates a major variable,
namely the donor pool.

Rational Design of Recombinant Human Hemoglobin

Expression systems were developed which resulted in the coproduction
of both the α- and ß-globin chains of hemoglobin within the same cell.
The co-factor heme is incorporated into properly folded globin chains,
which assemble themselves into fully functional hemoglobin
tetramers. The intracellular hemoglobin is produced as a soluble
protein. Model expression systems were developed in *E.coli*[7] and
yeast[8].

A genetic modification of the hemoglobin molecule was necessary to
solve the problem of high oxygen affinity associated with cell-free
hemoglobin. Fortunately, nature has provided a number of mutants
which suggested potentially useful genetic modifications of the
hemoglobin molecule. In addition, an analysis of the x-ray crystal
structure for hemoglobin suggested other potential mutants with lower
oxygen affinity. Somatogen examined and produced many
hemoglobin mutants, and ultimately chose one commonly known as
hemoglobin Presbyterian. This mutation is a single amino acid
substitution (asparagine to lysine) on the hemoglobin molecule at
position 108 of the β-globin chain.

The next step in modifying recombinant human hemoglobin for use as a HBOC was to solve the problem of the cell-free hemoglobin tetramer dissociating into $\alpha\beta$ dimers. Somatogen's collaboration with the Medical Research Council Laboratory of Molecular Biology facilitated the rational design of a genetically engineered solution to this problem. Examination of the x-ray crystal structure of hemoglobin revealed that the amino-terminus of one α-globin chain lies close in space (3 - 7 Å) to the carboxy-terminus of the other α-globin chain. Somatogen evaluated several different strategies to span this distance, ranging from a direct linkage of the two α-globin chains to the insertion of amino acid bridges consisting of several amino acids. The ultimate solution was to insert a single amino acid (glycine) between the space, producing a fusion protein or a di-α-globin chain. Covalent bonding of the two α-globin chains prevents dissociation into $\alpha\beta$ dimers.

The engineered hemoglobin molecule, termed rHb1.1, contains both the di-α-globin modification and the single amino acid modification on the β-globin chain. Initial animal and laboratory studies showed that this molecule was indeed a viable candidate for clinical trials as a HBOC[9]. The molecule has a P_{50} of 30 - 33. Oxygen equilibrium curves for fresh whole blood, hemoglobin isolated from red cells (HbA$_o$) and recombinant human hemoglobin (rHb1.1) are compared in Figure 1. We have shown in animal studies that the di-α modification eliminates the renal toxicity associated with HbA$_o$ and extends the plasma half-life[9]. The next step in the development of rHb1.1 has been to study its effects in human clinical trials.
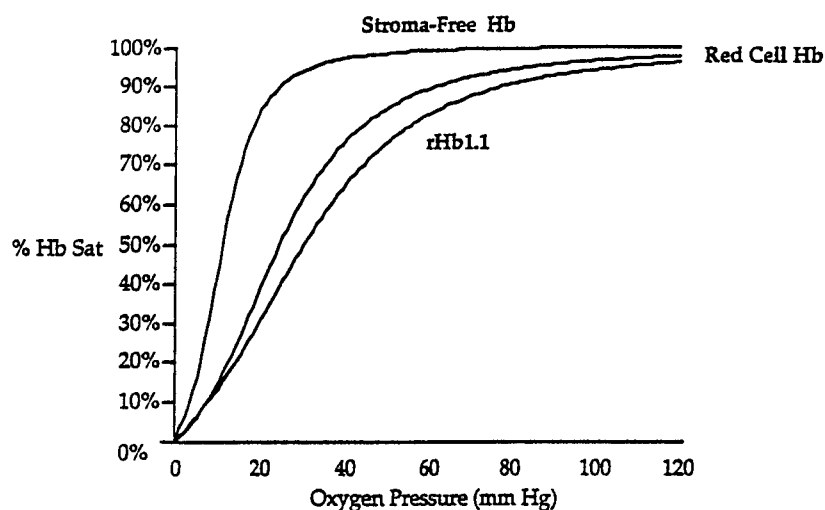
Figure 1

Oxygen equilibrium curves

Oxygen equilibrium curves are shown for fresh whole blood, HbA_0 isolated from red cells and recombinant human hemoglobin, rHb1.1. The curve for whole blood was measured at 37° C. The curves for cell free HbA_0 were measured at 37° C in 50 mM HEPES buffer, pH 7.4, containing 100 mM NaCl.

Initial Clinical Experience with Recombinant Human Hemoglobin (rHb1.1)

The primary objective of the initial Phase I studies was to establish the safety profile of rHb1.1 in normal male volunteers. The subjects in these initial studies were dosed with rHb1.1 at four dose levels, ranging from 0.015 g/kg to 0.11 g/kg. The maximum rate of infusion was 2.75 mL/kg/hr. As a result, the largest dose of rHb1.1 was administered in 48 minutes. Human serum albumin (HSA) was used as a control

solution. There were a total of 24 subjects in these first 4 dose levels.
Four subjects in each dose group received rHb1.1 and two subjects in
each dose group received HSA. Therefore a total of 16 subjects received
rHb1.1 and 8 subjects received HSA.

All subjects were initially screened by physical exam and laboratory
studies to establish baseline values. At the time of infusion, the
subjects were monitored with pulse oximetry and single lead ECG, and
had repeated vital signs measured. Subjects were not orally or
intravenously hydrated during the study. Subjects were monitored in -
house during the first 24 hours post-infusion. Symptoms were
assessed, vital signs were measured, and laboratory studies were
repeated. The subjects were then discharged and followed for 56 days,
with repeated clinical assessment and laboratory evaluation.

The laboratory data collected on these subjects included hematologic
parameters, serum chemistries, arterial blood gases, direct and indirect
Coombs, various coagulation studies, complement levels, routine
urinalysis, and specific measurements of renal function (see Table 1).
There were no clinically significant laboratory abnormalities associated
with the administration of rHb1.1 in these initial subjects.

Traditionally, researchers developing HBOCs have had concerns about
potential liver, kidney and lung toxicities. In Somatogen's initial
studies, there was no evidence of renal impairment. Serum creatinine
values were normal, as were creatinine clearance measurements made
after infusion. There was no appreciable hemoglobin excretion in the
urine. Urine dipsticks were negative in 14 of 16 subjects receiving

Table 1

Clinical chemistries performed in Phase IA study

- Hematology
  - CBC
  - Differential cell count
  - Erythrocyte sediment rate
  - Erythrocyte fragility
- Serum chemistries
  - Electrolytes
  - Creatinine
  - Liver function tests
  - Amylase
  - CK
  - Serum iron
  - Transferrin
  - Ferritin
- Serum complement
- Arterial blood gas
- Direct and indirect Coombs
- Coagulation Studies
  - PT, PTT
  - Fibrinogen
  - Fibrin D-Dimers
- Urinalysis
- Urine chemistries
  - Creatinine clearance
  - NAG
- Antibody levels

rHb1.1. Two subjects receiving rHb1.1 exhibited trace levels. A trace level, as measured by dipstick, indicates a maximum excreted hemoglobin level of 1 µg/mL. This assay cannot distinguish between HbA_o, free heme or rHb1.1. There was no evidence of any hepatic dysfunction, as measured by albumin, total protein, ALT, AST, and total bilirubin. There were no pulmonary adverse events. Blood gas measurements were normal.

Increases in blood pressure have been reported with other cell-free

HBOCs. Comparison of the maximum systolic and the maximum diastolic pressure at each dose level for each subject over 24 hours post - infusion in these initial studies revealed no consistent differences between the rHb1.1 and the HSA groups. In addition, comparison of the lowest dose to the highest dose of rHb1.1 revealed no dose-response effect in the maximum systolic and diastolic blood pressure levels. Thus, there were no treatment-related blood pressure effects observed in the initial 16 subjects receiving rHb1.1 at these four dose levels, when compared to the 8 subjects receiving HSA. Blood pressure effects will be a subject of scrutiny during further dose escalation because of historical concerns.

There was one symptom complex which was associated with rHb1.1. The complex occurred 3 - 8 hours after infusion in some of the first 12 rHb1.1 subjects and included fever (defined as a temperature greater than 38° C), chills, headache, and myalgias. In the subjects exhibiting this complex, there were some associated laboratory findings which represented changes from baseline but were generally not out of the normal range. These findings suggested an acute phase response. All of the symptoms resolved either spontaneously or after oral ibuprofen administration (400 - 600 mg).

Traditional animal and laboratory testing did not predict these signs and symptoms of pyrogenicity. Additional assays, both *in vitro* and *in vivo*, which were both more predictive and more sensitive for this response were developed by Somatogen during the course of the clinical trials. A minor process change was subsequently implemented in a chromatography step during the purification steps of rHb1.1

manufacture. Following this process change, 4 additional subjects received rHb1.1. None of these subjects receiving rHb1.1 manufactured after the minor chromatography process change developed a fever.

In summary, in initial studies rHb1.1 in doses up to 0.11 g/kg administered over 48 minutes produced no clinically significant blood pressure changes and no organ toxicity. There were no clinically significant laboratory abnormalities associated with the administration of rHb1.1, including activation of complement or other "effector systems". The symptoms of fever, chills, mild headache, and myalgia, observed initially, were easily managed with oral ibuprofen. A minor manufacturing process change appears to have reduced both the occurrence and the severity of this symptom complex.

Somatogen has used the tools of recombinant DNA and genetic engineering to rationally design a recombinant human hemoglobin molecule, rHb1.1. This molecule, after initial human testing, appears to be a promising candidate for a hemoglobin-based oxygen carrier. Further animal and human studies with rHb1.1 are warranted.

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STABILIZED HEMOGLOBIN VESICLES

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ABSTRACT

The Hb-vesicles which encapsulate the purified and concentrated Hb more than 40 g/dl with a uni- or bi-lamellar membrane are prepared by extruding the dispersion of mixed lipids through membrane filters (final pore size: 0.2 µmφ). They transport large amount of oxygen with satisfying rheological properties such as oncotic pressure and solution viscosity. Oxygen affinity of the Hb-vesicles is adjusted so as to exceed the ability of oxygen transport of human blood by coencapsulating allosteric effectors in the Hb-vesicles. The solution is sterilizable because of the diameter of Hb vesicles less than 0.2 µmφ. The Hb-vesicles are stabilized by using polyphospholipid or glycolipid as membrane components. No change in oxygen affinity and particle size was confirmed during long time storage at 4 °C. The stabilized Hb-vesicles can also be stored as frozen or dried state. The dried Hb-vesicles are regenerated by simply adding pure water. Simple *in vitro* test indicates that Hb-vesicles have the reduced inhibitory action of Hb to the EDRF-mediated vasorelaxation.

INTRODUCTION

Hb-vesicles are the concentrated SFHb encapsulated with thin lipid membrane like a red cell. They have many advantages which are expected to overcome the problems of the blood substitutes based on modified Hb.

Advantages of the Hb-vesicles come from the encapsulation of concentrated Hb with thin phospholipid membrane. Oncotic pressure is very low and

467

adjustable to human blood(ca. 25 Torr). Solution viscosity is also lower than blood (ca 4.5 cp) and adjustable. Oxygen affinity of Hb is controlled by coencapsulating allosteric effectors. The advantage of none chemically modified Hb is quite obvious. Furthermore, direct contact and actions of stripped Hb to tissues are reduced by this encapsulation.

However, the following issues have been remained to be unsolved in conventional Hb-vesicles. (i) Conventional Hb-vesicles have particle size of ca. 0.8 - 0.4 μmϕ and large size distribution [1-3]. Therefore, sterilization, for example, with 0.2 μmϕ pore size membrane filter is impossible. (ii) The phospholipid vesicles disappear from blood stream through fast RES clearance, which is relating to the particle size and dose amount. Particle size less than 0.2 μmϕ with maintaining the inside high Hb concentration is essential for Hb-vesicles. In addition, reduction of the administered lipid amount is an important factor to reduce the influence of vesicle on RES. The development of encapsulation techniques will be described in detail at another paper on this symposium. (iii) Aggregation and fusion of Hb-vesicles and leakage of Hb occur by long term standing or outer stimuli, since a phospholipid vesicle is an assembly of lipids by hydrophobic interaction. This instability of Hb-vesicles *in vitro* and *vivo* is expected to be overcome by using two kinds of special lipids. One is polyphospholipid obtained by polymerization of unsaturated phospholipid as Hb-vesicles [4-6]. The phospholipid molecules are connected by covalent bond to promise high structural stability of the vesicle. The other is glycolipid having oligosaccharide chain [7, 8]. The saccharide chains extend upon the surface of Hb-vesicles and prevent the aggregation of vesicles. The vesicles covered with oligosaccharide chains show high stability even in dry state by hydrogen bond between saccharide chain and vesicular surface.

This paper describes the preparation and performances of stabilized Hb-vesicles from engineering side.

MATERIALS AND METHODS
Purification and adjustment of Hb

Firstly, HbO_2 was converted to HbCO in RBC prior to purification procedure because HbCO is stable against oxidation and denaturation of Hb during solvent treatment and heating [9]. Then, hemolysis was carried out by mixing RBC with organic solvents such as dichloromethane or hexane without dilution of the Hb

solution. The organic solvent layer with stromata was separated easily by mild centrifugation (1,900g, 15 min). The resulting hemolysate was heated at 60 °C for more than 1 hour in order to denature water-soluble proteins other than HbCO. At the same time, pasteurization is also performed if necessary. After separation of the denaturated proteins by centrifugation (1,900g, 20 min), purified Hb of which concentration about 25 g/dl was obtained. Water-soluble low molecular weight compounds were removed by dialysis, and then osmolarity, pH and other properties of the Hb solution were adjusted by additives.

The HbCO was concentrated to ca. 40 g/dl with a hollow fiber module of which cut off molecular weight was less than 30,000, and allosteric effector was added to the Hb solution.

Preparation of Hb-vesicles

Phospholipid, cholesterol, and fatty acid are mixed in an organic solvent at a mixing molar ratio of 7/7/2 and freeze-dried from benzene. For the stabilized Hb-vesicle systems, polymerizable unsaturated phospholipid was used, or glycolipid was added to the lipid mixture in organic solvents [8]. The powder was added to a HbCO solution (ca. 40 g/dl) and dispersed by mechanical mixing at 4 °C. The resulting large multilamellar vesicles were converted to uni- or oligolamellar vesicles as changing the pore size of polycarbonate membrane filter until 0.2 $\mu m\phi$ [10]. Hb molecules which were not encapsulated were removed by dialysis with a hollow fiber module. Decarbonylation of HbCO was carried out after the preparation of Hb-vesicles. HbCO is easily and completely converted to HbO_2 in the Hb-vesicle by irradiating visible light onto the hollow fiber module, and sterilized air passes through outer side of the fiber and the Hb-vesicle dispersion through the inner side. The procedure was completed within 20 min. Concentration of the Hb-vesicles was performed by using the same hollow fiber module. The Hb-vesicles with unsaturated phospholipid were polymerized by γ-ray irradiation (total dose: 0.75 Mrad, 4 °C) [4, 5].

Characterization of Hb-vesicles

The size distribution was measured with a particle analyzer (COULTER N4-SD) and transmission electron micrograph (negative stain method). The oxygen-binding curves were obtained with a Hemox-analyzer (TCS-Medical Products) in a Hemox solution (pH7.4, 37 °C). Leakage of Hb from the Hb-vesi-

cles was calculated by Hb concentrations before and after subjecting a Hb-vesicle dispersion to gel permeation chromatography.

Inhibitory Activity of EDRF-mediated Vasorelaxation [11]

Thoracic aortic strips of 3 mm in width were prepared from New Zealand albino rabbits of either sex, 2.0-2.5 kg, and mounted in an organ bath under 1 g of tension in a Tyrode solution (37 °C) of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 2.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.6 mM, pH7.4 aerated with room air. Changes in isomeric force were recorded on a polygraph. The tissues were precontracted with phenylephrine (PE, 1 μM), and acetylcholine (ACh, 1 μM) was added to elicit a steady-state relaxation. Concentration-response curves to Hb were then constructed by adding cumulative concentrations of Hb solutions (from 10 ng/ml to 1 mg/ml). The response to each concentration was expressed as a percentage of the maximal relaxation induced by ACh.

RESULTS AND DISCUSSION

Purification of Concentrated Hb

Protein purity of our purified Hb was 99.95 %, and the percentages of residual phospholipids were less than 0.2 % [9]. The metHb was less than 1.0 %. No change was observed in the oxygen-binding curves before and after purification.

The Hb solution concentrated above 40 g/dl shows the excellent passing ability through membrane filters with pore size of 0.2 μmφ, that proves the high purity and is very important for the preparation of Hb-vesicles with the following extrusion procedure.

Properties of Hb-vesicles

The size of Hb-vesicles was reduced to 0.2 μmφ by extruding the dispersion of lipid mixture in a concentrated Hb solution through 0.2 μmφ pore size polycarbonate membrane filter. The resulting vesicles had a mean diameter of 195 ± 37 nmφ (ca. 0.2 μmφ) with narrow size distribution. This Hb-vesicle dispersion is sterilizable by filtrating it through 0.2 μmφ pore size filter before use. Concentration of endotoxin in the final product measured by chromogenic assay

with a TOXICOLOUR system (Seikagaku Co., Japan) was negligible (< 0.01 EU/ml). The HbCO was less than 1.0 % after decarbonylation.

Oxygen affinity of the encapsulated Hb is easily controlled by coencapsulating an allosteric effector such as pyridoxal 5'-phosphate and ions such as chloride ion and proton. On the assumption of oxygen-transporting efficiency (OTE) as the difference in oxygen-binding percentages between the oxygen partial pressure of 110 Torr at lung and 40 Torr at mixed vein, this value is estimated from oxygen saturation curve, namely P_{50} and Hill coefficient. Normal red cells with P_{50} (28 Torr) and Hill coefficient (2.8) to yield OTE 28 %. In our Hb-vesicles, P_{50} and OTE could be adjusted not only exactly the same as those of RBC, but also superior to RBC. For example, P_{50} values were from 35 to 40 Torr, and Hill coefficient around 2.2. OTE was more than 40 % as shown in FIG. 1.

Oxygen transporting amount of Hb-vesicles (ml/100ml) is determined from the equation (1).

O_2 Transport of Hb-vesicles (ml/100ml) = [bound O_2 (ml)/Hb(g)]
       x oxygen transporting efficiency (%) x ([Hb]/[Lipid]) x [Lipid]   - - - (1)

The unit of concentration is (g/100 ml). Oxygen transporting efficiency was optimized by coencapsulating allosteric effectors. The [Hb]/[Lipid] ratio is determined from the Hb concentration in the interior of Hb-vesicle and the number of bilayer membrane. The Hb concentration used in the experiment is as high as possible. Therefore, the [Hb]/[Lipid] ratio is owing to the number of bilayer membranes. Unilamellar Hb-vesicle is an ideal structure, which enables to reduce the dose amount of lipids significantly. The number of bilayers was controlled by the conditions of the feed Hb solution such as ionic strength, pH, temperature, etc., because Hb and lipids are kinds of electrolytes. The Hb-vesicle having the [Hb]/[Lipid] ratio of 2.4 was obtained. This means the encapsulation of about 40 g/dl of Hb within the unilamellar vesicles.

Solution Properties

Oncotic pressure of a SFHb solution is relatively high and exceeds that of human blood (ca. 25 Torr) when the concentration of Hb is above 8 g/dl. On the other hand, that of Hb-vesicles themselves is less than 1 Torr because about 14,000 Hb molecules are incorporated into the inside of one vesicle. Additionally,
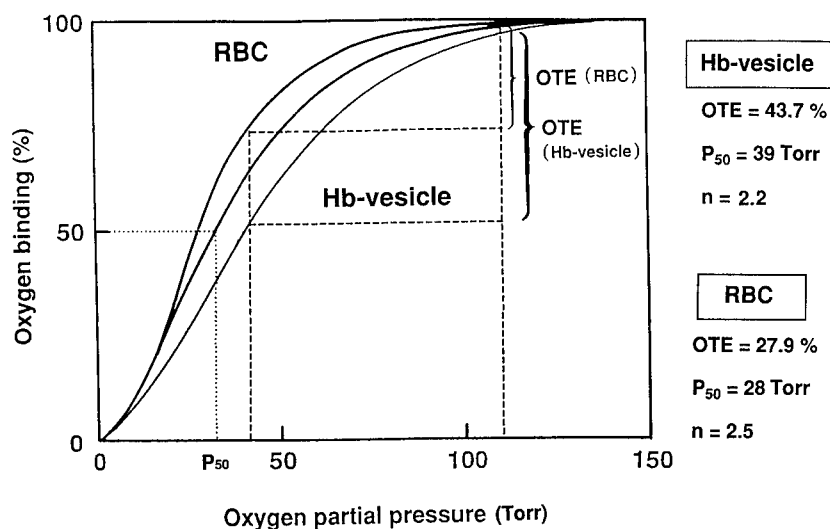
FIGURE 1
Oxygen binding properties (37 °C, pH 7.4) of Hb-vesicles controlled by coencapsulation of allosteric effectors.

oncotic pressure of Hb-vesicles is easily adjustable to that of human blood by additives such as albumin (5.6 wt%) or dextran with average molecular weight of 40,000 (2.2wt%).

Solution viscosity of human blood is about 4.5 cp. While, the viscosity of a Hb-vesicle dispersion is very low and increases slightly from 1 to 2 cp with the concentration of vesicles. Even after albumin, for example, was added to adjust the oncotic pressure to 25 Torr, the solution viscosity of Hb-vesicles is still lower than that of human blood. These solution properties are advantages of the Hb-vesicles.

Stability of the Stabilized Hb-vesicles

Storage at 4 °C or in frozen state: Oxygen binding properties of Hb-vesicles (P_{50}, n, and OTE) were maintained during long term storage at 4 °C over 2 months [4]. That indicates no leakage of allosteric effectors from the Hb-vesicle. However, 10 % of Hb was converted to met-Hb. But this metHb formation could be avoided by storing Hb-vesicles in the frozen state for such long term storage.

Excellent stability of our stabilized Hb-vesicles against freeze-thawing repetition guarantees storage in a freezer [4, 6]. No leakage of Hb and no change in the vesicular size was observed even after 10 freeze-thawing repetition, whereas the vesicular size and the leakage amount of Hb significantly changed after only one repetition for conventional Hb-vesicles.

Storage as freeze-dried powder: The dispersion of Hb-vesicles stabilized with polyphospholipid could also be freeze-dried [5], that had been estimated to be impossible for the conventional Hb-vesicles without the addition of disaccharides [12]. The powder was then dissolved into water as shown in FIG. 2. In the absence of sucrose, the particle size measured with a particle analyzer changed without leakage of Hb. It means that the polyphospholipid vesicles are stable even in the absence of sucrose but do not disperse spontaneously and completely. Therefore, a small amount of sucrose (50 mM) was added to the dispersion of polyphospholipid vesicles before freeze-drying in order to enhance the dispersion of the freeze-dried powder. In the case of conventional Hb-vesicles, more than 80 % of Hb leaked out with the particle size distribution becoming broader.

Storage as desiccated flakes: To the dispersion of Hb-vesicles modified with oligosaccharide by glycolipid, 100 mM of sucrose was added. It was desiccated over P_2O_5 and then dried *in vacuo* to yield flakes of Hb-vesicles. The Hb-vesicles without glycolipid leaked 11 % of Hb after redispersion of the flakes. While the incorporation of 8 mol% of glycolipid reduces Hb-leakage less than 3 %. Size distribution was also preserved effectively by glycolipid. The stability is probably caused by effective covering of the vesicular surface with the oligosaccharide chains through hydrogen bond.

Suppression of the aggregation of Hb-vesicles by glycolipid: The mixtures of phospholipid/cholesterol/negatively-charged lipid are usually used to provide stable vesicles. However, this kind of vesicles easily aggregates in the presence of Ca^{2+} ion because Ca^{2+} binds on the negatively-charged surface and crosslinks vesicles [8]. As shown in FIG. 3, an increase in the viscosity at low shear rates in the presence of Ca^{2+} means the aggregation of vesicles. The Hb-vesicles with glycolipid showed little increase in the viscosity. The same profiles were also observed after the addition of dextran. The Hb-vesicles containing
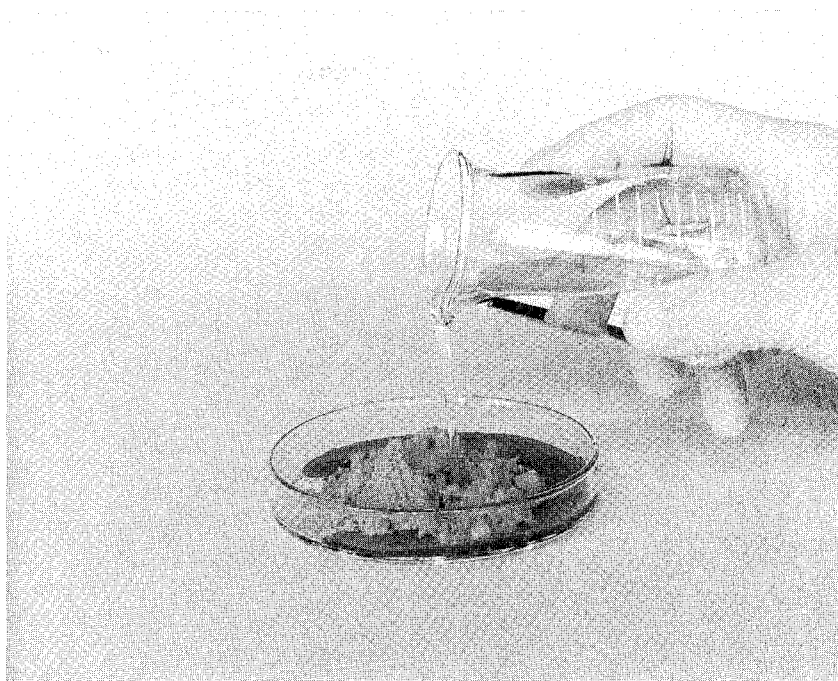
FIGURE 2
A picture of the powder of the stabilized Hb-vesicles(polyphospholipid).

glycolipids maintained the low viscosity in comparison with the vesicles without glycolipids. This suppression of vesicular aggregation is considered to be that the oligosaccharide chains extending form the surface exclude access of the vesicles.

Reduction of EDRF-mediated Vasorelaxation [11]
        Endothelium-derived relaxing factor (EDRF) is considered to be nitric oxide (NO), and the abnormal elevation of blood pressure after the administration of modified Hb attributes to the high reactivity of stripped Hb with NO released from endothelium cells.
        On the other hand, RBCs which also contain high concentrated Hb do not cause such a problem. The difference seems to be due to the encapsulation of Hb
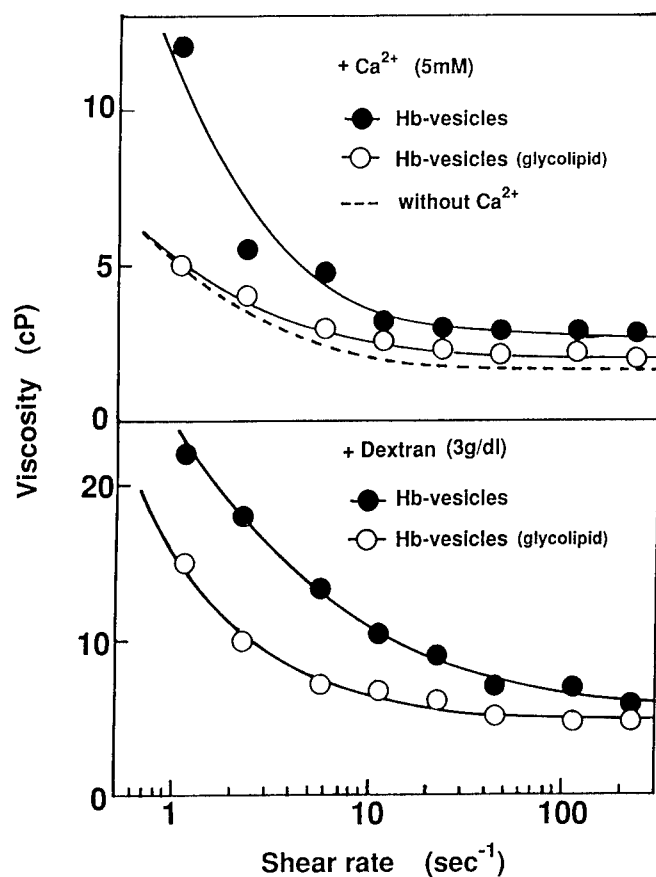
FIGURE 3
Stability of Hb-vesicles (glycolipid) against the aggregation of vesicles after the
addition of Ca^{2+} or dextran ($\overline{M}n. = 40{,}000$).

within a vesicle. The *in vitro* inhibitory activity of Hb-vesicles to EDRF-induced
vasorelaxation in comparison with stripped Hb and RBCs was studied under the
same Hb concentration. In all cases, the inhibitory action of vasorelaxation ap-
peared, but the Hb concentration to yield the action was different in each sample.
FIGURE 4 clearly indicates the effect of Hb encapsulation. Encapsulated Hbs,
namely both RBCs and the Hb-vesicles, show nearly hundred times less inhibitory
activity to ACh-induced relaxation.
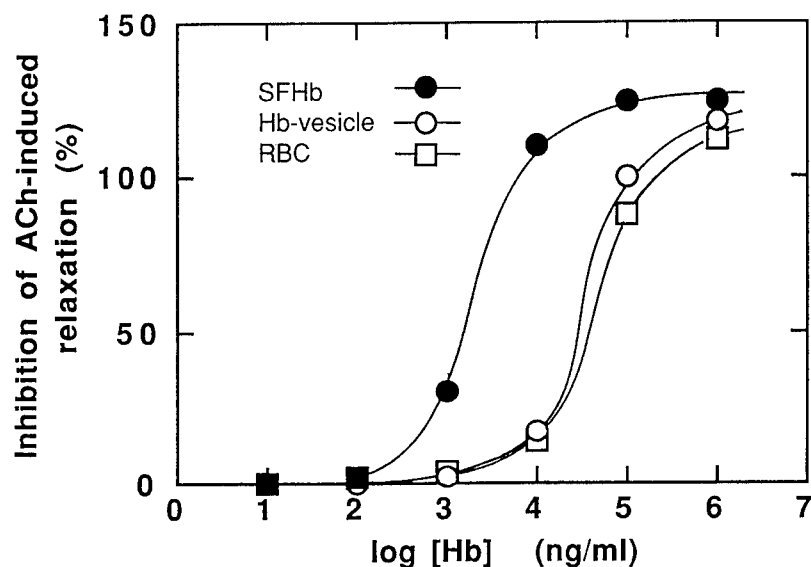
FIGURE 4
Inhibition of ACh-induced relaxation by the addition of solutions containing Hb.

CONCLUSION

The Hb-vesicles with high quality are prepared by extrusion. The properties of Hb-vesicles are: (i) oncotic pressure and solution viscosity of the Hb-vesicle solution are low and adjustable to human blood, (ii) the Hb-vesicles using polyphospholipids or glycolipids show excellent stability against freezing or drying, (iii) the Hb-vesicles show less inhibitory activity to EDRF-mediated vasorelaxation to the same value as RBC.

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CROSS LINKING ENCAPSULATED HEMOGLOBIN WITH SOLID PHASE SUPPORTS: LIPID ENVELOPED HEMOGLOBIN ADSORBED TO SURFACE MODIFIED CERAMIC PARTICLES EXHIBIT PHYSIOLOGICAL OXYGEN LABILITY

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ABSTRACT

To reduce hemoglobin toxicity, a cross linking agent is generally used. To preserve hemoglobin function, an allosteric modifier is generally used. Historically, the use of one has precluded the use of the other. A potential solution to this problem was investigated.

Hemoglobin A_0 was adsorbed irreversibly to carbohydrate coated nano-sized diamond particles and then encapsulated in a standard mixture of phospholipids. Endotoxin free preparations with concentrations of bound hemoglobin near 10 g/dl were achieved with as little as 0.1 g/dl of free hemoglobin and remained stable over a 48 hour period. By transmission electron microscopy these particles appeared roughly spherical and measured approximately 75 nanometers well below that of alveolar capillary vessels. In shallow pH gradients, liquid electrophoresis demonstrated that such constructs exhibit Bohr effect behavior by the induction of a dramatic surface charge inversion at around pH 6.8. To evaluate oxygen lability, oxygen saturation trials were conducted in isosmolar physiological salts. Normal sigmoidal binding behavior of O_2 over a typical pO2 gradient could be modulated by systemic levels of pyridoxyl-5-phosphate. Constructs with a P_{50} as low as 12 mm Hg could be increased to 37 mm Hg with the allosteric effector. Viscosity and bio distribution studies are to follow.

The use of solid phase nanocrystalline supports to cross link hemoglobin may reduce the toxicity of free hemoglobin while still enabling the use of allosteric modifiers.

INTRODUCTION

The demand for red cell surrogates, or artificial blood, is driven by a number of clinical concerns. For transfusions and resuscitation, natural blood carries the risk of infection, incompatibility, poor shelf life stability, and a relatively limited supply. Alternatives to natural blood are therefore being explored.

The approach described below was inspired by a physicochemical perspective of the hemoglobin molecule. Blood, after all, is a dispersion of formed elements in an aqueous colloid. Erythrocytes are the principle formed elements and provide the life sustaining function, in conjunction with the heart, lungs, blood vessels, and kidneys; of transporting and protecting the oxygen-carrying pigment, hemoglobin, to the tissues. The oxygen-binding properties of hemoglobin are sensitive to factors such as the cooperative effects of O_2 binding, pH and CO_2 levels, and the presence of other metabolic intermediates such as 2,3-diphosphoglycerate. The synergistic effects of these factors produce a well-known sigmoidal curve plot of the relationship between oxygen affinity and the partial pressure of oxygen (pO_2): there is high oxygen affinity in the lung where the pO_2 is high, and a low oxygen affinity in the tissues, where the pO_2 is low. The uptake and delivery of oxygen by hemoglobin is associated with allosterism of the hemoglobin molecule.[1]

Over three years ago, we developed a surface modified nanocrystalline technology suitable for delivering a wide array of biologically active molecules.[2] In this communication, we describe our investigation of the ceramic system for delivering hemoglobin in the guise of a red cell surrogate.

MATERIALS AND METHODS

Briefly, fine ceramic particulates comprised of pure carbon in the nanometer size range, following surface modification with carbohydrates (disaccharides) to reduce both surface charge and surface energy, are dispersed in an aqueous medium. Hemoglobin is allowed to adsorb and the construct is then sealed with a lipid film. The product is a 50-100nm lipid encased solid supported hemoglobin carrier in which hemoglobin is surface immobilized yet exhibits physiological oxygen binding affinity.

Disaccharide coating. One (1.00) g. of acid cleaned commercial ultra fine synthetic diamond particles (GE Series 300, Worthington, OH) was dispersed in 5.0 ml of a 100mM cellobiose (Sigma) solution with 175 watt sonication (Branson) for 10 minutes. The colloid was then incubated at 4.0 °C overnight in a 10 kD stir cell. The following day, this colloid was lyophilized for 24 hours and reconstituted in 1.0 ml of double distilled H_2O. Unadsorbed cellobiose was removed by 10 kD stir cell ultra filtration (UF)

(Filtron) against 100 ml of 20 mM phosphate buffer (pH 7.4) (PRB) and corrected to 2.0 ml.

Attachment of hemoglobin. Four ml [0.124 g/ml] were then added to 1.0 ml of human hemoglobin[[26.10 g/dl as calculated by a standard calibration curve] and then the colloid was slowly dialyzed into 100 ml of 0.5X phosphate buffer overnight at 4.0°C under a nitrogen head of 20 psi and a dialysis filter of 10kD.

Lipid coating. The product was then coated with a 1:1:0.1 molar ratio of phosphotidyl choline, phosphotidyl serine, and cholesterol, respectively.

Physical characterization of the red cell surrogate was performed by measuring surface charge across a wide pH range. Size was measured by transmission electron microscopy. Hemoglobin binding was measured by immunogold affinity. Hemoglobin concentration and oxygen binding affinity was measured spectrophotometrically.

RESULTS

Doppler electrophoretic light scatter analysis of the zeta potential (surface charge) showed stable attachment of the hemoglobin to the carbohydrate modified diamond core over a broad pH range (FIGURE 1). Hydrogen ion interactions at low pH's (Bohr effect) were noted. Repeated measurements of the colloid stored at 4°C for up to seventy days and 37°C for up to thirty days showed no change in surface charge . Transmission electron microscopy demonstrated particles in the 50-100 nm size range with approximately 2% unbound hemoglobin. Spectrophotometric analysis indicated a hemoglobin concentration of approximately 13 g/dl. Oxygen affinity curves of prototype constructs showed a p50 of 26-32 torr (FIGURE 2). *In-vivo* testing is pending.

DISCUSSION

Stroma free, cross linked, liposome encapsulated and now surface immobilized hemoglobin are four competing hemoglobin based technologies. Stroma-free hemoglobin is potentially problematic because of renal and coagulation toxicity.[3,4] Recently, these complications have been attributed to contaminants such as phospholipids and endotoxins and stroma free hemoglobin is again receiving attention. A relatively pure, disease-free source of bovine hemoglobin shows some promise as a blood substitute.[5,6,7,8] Stroma-free hemoglobin contains no 2,3-diphosphoglycerate, and consequently such preparations exhibit elevated oxygen affinities (P_{50} of 12 mm Hg) in comparison to whole blood (P_{50} of 26 mm Hg) which can impair the ability of these hemoglobin solutions to deliver oxygen to tissues.

IMMOBILIZED HEMOGLOBIN/SURFACE CHARGE
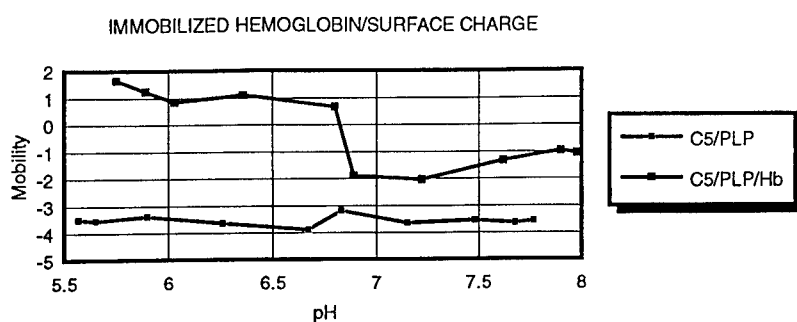
FIGURE 1 PLP (P5P) acts as an allosteric effector to Hemoglobin. This is evidenced by surface charge data of Diamond (C5)/P5P and Diamond (C5)/P5P/Hemoglobin particles. At pH~6.8, a huge drop in mobility (y axis units of $\mu m \cdot cm/V \cdot s$) occurs   in the C5/P5P/Hemoglobin samples while at the same pH a sharp increase in mobility occurs with the C5/P5P samples. Thus it is postulated that at pH 6.8, a conformational change in the P5P occurs which physically changes the conformation of the Hemoglobin.

% Sat vs pO2
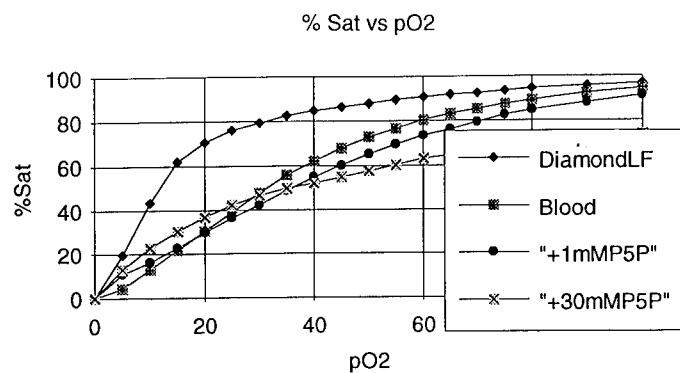
FIGURE 2 Spectrophotometric measurements of the oxygen binding affinity of red blood cell surrogates based on surface modified nanocrystalline ceramics. Note that the curves for whole human blood and for synthetic blood in the presence of the allosteric modifier pyridoxyl 5 pyrophosphate (+30mMP5P) are virtually identical.

Cross linking hemoglobin has been one approach to solving the renal toxicity problems. Hemoglobin preparations containing the cross-linking molecule pyridoxyl 5'-phosphate exhibit reduced oxygen affinity, but also exhibit a shorter half life. Reported complications of pyridoxylated polyhemoglobin administration in resuscitation and isovolumetric exchange experiments include complement activation, reticuloendothelial inhibition, immunogenicity, and vasoconstriction.

Recent efforts to synthesize a biocompatible and effective blood substitute have turned to liposome encapsulation as a method for producing artificial red blood cells. Liposomes technologies so far seem to be free of many of the complications which perfluorocarbon and free hemoglobin technologies have experienced including immune sensitization, renal damage or hepatic trapping, cytotoxicity, hyperosmolarity, and unnatural oxygen affinities leading to decreased oxygen delivery to tissues. Remaining complications include significant reticuloendothelial uptake, induction of platelet aggregation, possible suppression of the reticuloendothelial system and hemoglobin oxidation. Incorporating the ganglioside G_{M1} ($II^3NeuAc-GgOse_4Cer$) in low concentrations (at least 7 mol%) into the liposome membrane appears to prolong circulation times and reduce reticuloendothelial cell uptake.[9]

The surface modified nanocrystalline supports represent a new approach to producing red cell surrogates. The nanoparticles are small and thus should be able to evade the reticuloendothelial system. The surface immobilization property is a novel way to effect cross linking. By modifiying the surfaces with carbohydrates, the denaturation that would normally occur upon surface adsorption is blocked. A biophysical explanation for the mechanism by which disaccharides are able to stabilize the conformation of complex molecules such as proteins during the displacement of water has been offered by Crowe et al.[10] Briefly, they argue that certain sugars may replace the water around polar residues in membrane phospholipids and proteins thereby maintaining their integrity in the absence of water. It is a phenomenon that has been utilized by nature to protect anhydrobiotic organisms such as fungal spores, yeast cells, and cysts of brine shrimp against dessication.[11] It appears that sugars are capable of preserving the structure and function of both membrane bound and soluble proteins in the absence of water.

The family of sugars that exhibit dehydroprotectant properties are largely mono-, di-, and oligosaccharides, and their ability to do so correlates with their ability to form glasses.[12] Working with the model disaccharide trehalose, Green and Angell concluded that the trehalose/water system passes into the glassy state and thereby arrests all long-range molecular motion.[13] Denaturation is thus impeded. The glass transition temperatures of the most common natural dehydroprotectants, trehalose and sucrose, are

79°C and 70°C respectively.[14] The glass transition temperature of the disaccharide used by us, cellobiose, is 77°C. We therefore suggest that the surface modified nanocrystalline ceramics coated with the disaccharide cellobiose offer an epitaxial vitreous film that is not energetically bound to the hemoglobin but makes the hemoglobin much less mobile by viscosity effects in aqueous glassy solids. As such, the hemoglobin is bound loosely (without conformational alteration) but with sufficient binding energy (or viscosity) to prevent ready desorption. Hemoglobin is thus rendered conformationally stable and is able to exhibit the characteristic sigmoidal oxygen affinity property.

Last, the solid phase provides a nucleating site for the "liposome-like" lipid membrane converting the lipid addition process to one of surface film formation rather than liposome formation. As such, the red cell surrogates with solid phase cores are much smaller than the free formed liposomes. The ability of this material to act as a red cell surrogate *in vivo* is now under investigation.

ACKNOWLEDGMENTS

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EFFICACY, PHYSICAL PROPERTIES AND PHARMACO-KINETICS OF STERICALLY-STABILIZED LIPOSOME-ENCAPSULATED HEMOGLOBIN

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ABSTRACT

We recently reported that hemoglobin (Hb) encapsulated in liposomes (LEH) containing phosphatidyl-inositol (PI) was efficacious in rats. However, liposomes containing PI may temporarily compromise mononuclear phagocytic system (MPS) function. The objective of this study was then to determine whether a polyethylene oxide derivative of phosphatidyl ethanolamine (PEG-PE) would serve as an acceptable substitute for PI in our LEH formulation. In this study we compare the physical properties, pharmacokinetics and efficacy in life support obtained for Hb encapsulated with either PI or PEG-PE phospholipids. Both liposome compositions contained the same matrix lipids, egg derived phosphatidyl choline (PC) and cholesterol, were of similar size and contained the same amount of encapsulated Hb. The liposomes differed only in their phospholipid component, one containing 5 mol% PI and the other an equal amount of the sterically-stabilizing lipid PEG-PE. The physical characteristics of the PI and PEG-PE compositions were remarkably similar: only small amounts of

487

Met-Hb were generated during processing and following 1 month frozen storage, oxygen affinity and cooperativity and steady shear viscosity values for 30% by volume suspensions (in isotonic/isooncotic saline containing albumin) were near the normal values expected for whole blood, incubation in plasma at 37°C resulted in only small amounts of Hb release and shear had very little impact on Hb leakage. Circulation half-lives following 50% isovolemic exchange-transfusion in rats were also similar, about 15-20 hours for either formulation. Animals survived following 97% isovolemic exchange-transfusion of both compositions, confirming the efficacy of each.

INTRODUCTION

There is an urgent need for a universally transfusable (nonallergenic), oxygen-carrying blood replacement fluid that can be used in emergency situations to provide temporary life support until an adequate supply of whole blood becomes available. Numerous approaches have been taken in the attempt to develop materials which can deliver oxygen effectively and are safe for use as a red blood cell substitute; liposome-encapsulated Hb (LEH) has been developed more recently [1-9]. Liposome technology provides a mechanism for encapsulation and in vivo delivery of drugs, proteins, etc. which probably would otherwise be degraded, cleared rapidly, or toxic to the host [10].

The overall goal of our work is the development of a safe, efficacious and commercially viable oxygen-carrying red blood cell substitute composed of hemoglobin solution encapsulated in a liposome. In these studies "Stealth" liposomes (liposomes containing PI or polyethyleneglycol distearoyl phosphatidylethanolamine, PEG-PE) that are designed to evade recognition and rapid uptake by the (reticuloendothelial system (RES), however more recently referred to as the mononuclear phagocytic system) MPS [11-15] are used. Hemoglobin encapsulated in more conventional liposomes have been shown to provide an effective means of oxygen delivery in vitro and in experimental animals [7,16]. However, it was recently found that phosphatidylglycerol (from egg) liposomes bound to rat platelets, which was mediated by complement [17].

Although LEH containing PI have also been shown to be efficacious [18], our recent experiments [19] suggest that such systems, by overloading the MPS [20,21], cause alterations in phagocytic activity and increase host susceptibility to infectious challenge [22].   Our recent results with LEH containing PEG-PE suggest that they are less immunotoxic as they cause less adverse effects when treated animals were tested by infectious challenge [19]. It is the PEG-PE lipid component that is believed to be responsible for producing liposomes with surfaces which are sterically-stabilized such that e.g. plasma protein uptake is greatly reduced [23,36,38]. This study encompasses the further development, characterization and efficacy in life support of LEH using PEG-PE lipids.

MATERIALS AND METHODS

Human stroma-free hemoglobin solutions were prepared at 4 °C following aseptic techniques as described elsewhere [3,5]. All equipment used in processing Hb and LEH was depyrogenated and all water used (for example in the washing and lysing steps and in the preparation of phosphate-buffered saline) was sterile and pyrogen-free. To maximize oxygen-carrying capacity high concentrations of Hb solution up to 35 g% (i.e. 35 grams Hb per 100 ml solution) were used in some experiments to prepare liposomes. Pyridoxal-5-phosphate (P-5-P) (Sigma Chemical Co.) was added to the hemoglobin solutions to control oxygen affinity of the LEH to a value similar to that of fresh red blood cells. The antioxidant catalase, which acts as a scavenger of free radicals, was added to the Hb solution [24]. Also it is known that other constituents of the red blood cell hemolysate from which the hemoglobin solution is prepared protect against oxidation of hemoglobin and phospholipid [24].

The membrane lipids used to encapsulate Hb solution include partially hydrogenated egg phosphatidylcholine (PC, IV 40), cholesterol (CHOL), phosphatidylinositol (PI) or PEG-PE, and α-tocopherol (α-Tc). PC was from Asahi Chemical Company, Ltd., Tokyo, Japan and PEG-PE was obtained from Liposome Technology Inc. (Menlo Park, CA).   Other lipids were obtained from Sigma Chemical Co. (St. Louis, MO). All these materials were used as obtained without

further purification. The liposome membrane was formulated to contain the lipid molar ratios for PC:CHOL:PI or PEG-PE:α-T of 1.0:1.0:0.1:0.02. Both the PI and PEG-PE formulations were prepared at a lipid to Hb loading of about 150 μmols per ml of precursor Hb solution.

An aseptic double emulsion technique was followed in the preparation of LEH [18]. This method first involves the formation of a water-in-oil-in-water type multiple emulsion [25] using PC and cholesterol as primary and secondary emulsion surfactants, respectively, followed by organic solvent removal in a rotary evaporator operating under partial vacuum. As the organic solvents are removed, LEH spontaneously form in the excess lipid system. The evaporation procedure is continued until dryness using a single stage vacuum pump to maximize removal of all organic solvent and water so that the Hb concentration within the LEH is as high as possible. This results in the deposition of an apparently dry Hb/lipid thin film on the walls of the round bottom flask. Concentrated Hb solution is then added under agitation to rehydrate the lipid and form a coarse LEH suspension. Size reduction was achieved by homogenization using a Microfluidizer [18]. The LEH so formed were washed at least three times in isotonic phosphate-buffered saline (PBS) and centrifuged at 30,000 x g for 30 mins to remove all unencapsulated Hb solution and any residual organic solvent. Ideally, a finishing membrane extrusion step was used to ensure the final LEH suspension would pass through a 0.45μ sterilizing filter. Approximately 200 ml of the suspension was placed in a 400ml, 76mm diameter pressure filtration cell (Nucleopore, Pleasantville, CA) fitted with the appropriate pore size filter. Prior to addition of the LEH sample to the filtration cell, it was first prefiltered and then filtered under vacuum through 5, 3, and 1μ CF filters (Nucleopore). The resulting LEH samples was then reloaded into the pressure cell and using nitrogen as the pressure source (at pressures well below 100 psi) they were filtered through 0.8, 0.6 and 0.45μ CF filters; the last filter used is a sterile-grade 0.45μ filter. If desired all filtration steps can be done in the filtration cell and the LEH can

Oxygen content in terms of ml O_2 per ml of the LEH suspension sample was determined using a Lex-O_2-Con (Hospex Fiberoptics, Chestnut Hill, MA)

[28]. The Oxy-hemoglobin equilibrium dissociation and association curves for the two LEH preparations, measured as a function of oxygen partial pressure, were obtained using a Hemox-Analyzer (TCS Medical Products Co., Huntington Valley, PA). Oxygen affinity (P_{50}) and cooperativity (Hill exponent n) were determined from the generated curves. Encapsulated Hb concentration was determined by dissolving the liposome membrane with n-Octyl β-D-glucopyranoside detergent solution as described elsewhere [3,18]. The resulting Hb solution concentration was measured for oxy, reduced and met-Hb components by the method of Benesch et al. [26], modified using the extinction coefficient values provided by Van Assendelft and Zijlstra [27].

Steady shear viscosity of the suspension samples was measured in a uniform shear field with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40, Soughton, MA). Shear rates from 45 to 450 s^{-1} at 37°C were evaluated. The cone-and-plate geometry is very useful as it gives a good approximation of viscometric flow with constant shear rate throughout the flow field [29]. The stability of the LEH to shear, i.e. leakage of Hb, was evaluated by shearing the LEH samples for 30 mins in the viscometer as a function of shear rate. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma, both at 30% by volume. Following centrifugation (13,600 x g, Microcentrifuge Model 235C, Fisher Scientific) the concentration of total Hb in the supernatant of the sheared sample was compared with that of the unsheared sample. The benzidine method [30], which is known to be accurate down to concentrations of 1 mg/dl, was used for determining plasma Hb concentration at low concentration levels (i.e., in the mg/dl range) as a result of leakage of Hb from LEH. The effect of 1 day incubation at 37° on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma (both at 30% by volume LEH). Also the effect of cholesterol content in the liposome membrane on leakage of encapsulated Hb was evaluated.

Circulation half-life of LEH and efficacy in life support was evaluated on unconscious rats in Illinois Institute of Technology's Small Animal Lab. Female rats (Harlan Sprague, Indianapolis, IN) weighing 225 to 275g (8 to 12 weeks of age) were anesthetized using ketamine. Cannulation of the femoral artery and vein was carried out based on the model developed by Keipert and Chang [31]. Using a doubly cannulated rat and a peristaltic pump (Manostat, New York, NY), exchange transfusions were performed by removing blood at a constant rate of about 0.2 ml/min, coupled with its simultaneous isovolemic replacement with either LEH suspension or control. The decrease in hematocrit levels was recorded during the exchange-transfusion.

RESULTS AND DISCUSSION

Hemoglobin concentrations of up to 25g% were achieved in both PEG-PE and PI liposomes, representing greater than 80% of precursor Hb solution concentration. Possibly the other apparent 20% can be accounted for by the volume occupied by the lipid phase. LEH processing resulted in LEH containing about 0.9 μmoles encapsulated Hb per 82 μmoles of lipid (i.e. total lipid including cholesterol). These results were determined using a total phosphorous analysis in conjunction with Bligh-Dyer extraction of 1 ml samples of PEG-PE based LEH at a lipocrit of 30% containing an encapsulated Hb solution concentration of 20 g%. The phosphorous analysis was performed by LTI's quality control unit. Met-Hb generation accompanying LEH processing (for either lipid formulation) appeared to be small with only a 3% increase for encapsulated over precursor. These results correspond to an oxygen content for an LEH suspension sample (50% by volume LEH) of 15 volume% oxygen. Storage of PEG-PE based LEH for up to one month at -20°C resulted in percent met-Hb concentrations to levels of about 9%. Additions of various components to the lipid phase of LEH systems appeared to reduce the oxidative interactions between hemoglobin and membrane lipid. Some of these included the addition of cholesterol to the membrane phase to protect Hb from oxidation [32,33]. Also oxidation of Hb to met-Hb may have been inhibited by using partially hydrogenated PC instead of natural unsaturated

egg-PC [3]. Partial hydrogenation of egg PC to an iodine value of 40 as used here is known to convert the polyunsaturated fatty acids to monosaturated species which are far less susceptible to oxidation [34].

The size of the LEH containing PEG-PE in which thin-section electron micrographs were prepared was similar to that reported previously for liposomes containing PI [18]. LEH were passed 10 times through a Microfluidizer[TM] and resulted in a particle size range of a 3μ filtered sample from 50 nm to a little greater than 1μ with a median particle size of about 300 to 400 nm. The oxygen saturation curve obtained for LEH containing PEG-PE, the precursor Hb solution, and a whole blood sample using a Hemox Analyzer is shown in Figure 1. Oxygen affinity (based on P_{50}) and cooperativity (as characterized by the Hill coefficient) for the LEH suspensions appeared to be near the normal values seen for whole blood.

Steady shear viscosity results were obtained for PEG-PE and PI based LEH suspension samples (in PBS containing 7.5 g% albumin at isooncotic levels) for shear rates to about 500 s^{-1}. Although, viscosity results for both formulation suspension samples prepared at 50% lipocrit were higher than those obtained for human and rat whole blood (both at 45-46% hematocrit), viscosity results obtained for either LEH suspension sample of 30% lipocrit were slightly lower than that measured for the whole blood samples. All measurements were made at 37°C. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma, both at 30% by volume. Although leakage was highest for either formulation suspended in plasma, only 0.5% leakage of the encapsulated Hb was measured at the highest shear rate value tested, i.e. about 500 s^{-1}. The effect of storage of either LEH containing PEG-PE or LEH containing PI at 37°C on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma (both at 30% by volume LEH). Again, less than 0.5% leakage of the encapsulated Hb was observed for either LEH sample in plasma even after 24 hours of incubation (see Figure 2).
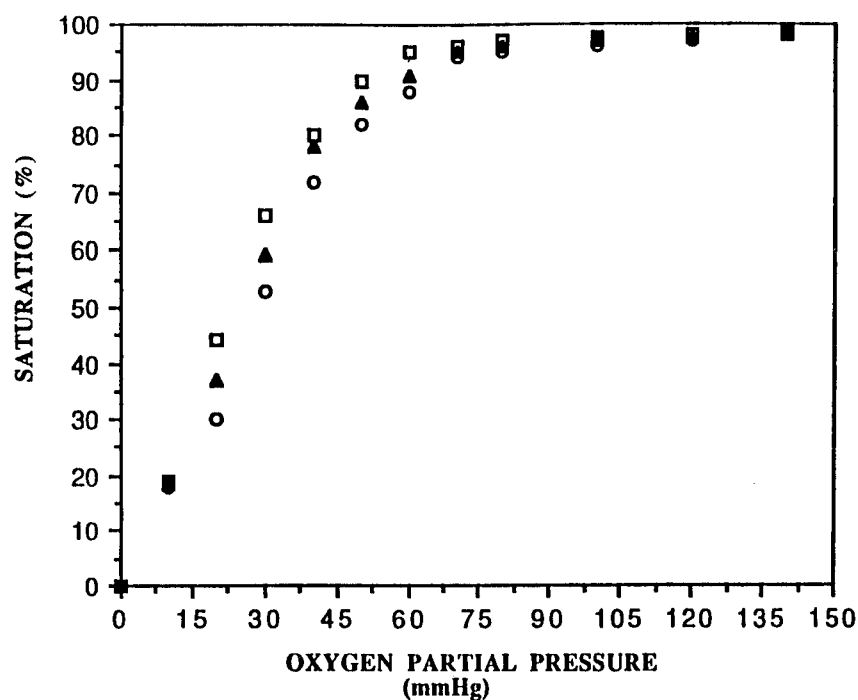
FIGURE 1.   Oxygen dissociation curve of precursor Hb solution △ ,
                    whole blood ○, and PEG-PE LEH □, measured at 37°C.

Circulation half-life following 50% isovolemic exchange-transfusion
typically was about 15 to 20 hrs for both of the formulation samples tested (see
Figure 3). These times are desirably long and compare very favorably to results
reported in another study for LEH containing dimyristoyl phosphatidylglycerol [5].
Also, high cholesterol content in the membrane, as is the case for both PEG-PE
and PI LEH formulations, prolongs liposome stability in circulation [35]. Other
recent studies have also found that PEG-PE significantly increased the blood
circulation time of liposomes formulated with it [11-15]. Also those studies have
shown PEG-PE liposomes to have greatly decreased uptake by the
reticuloendothelial system and thus enhanced circulation time compared to many
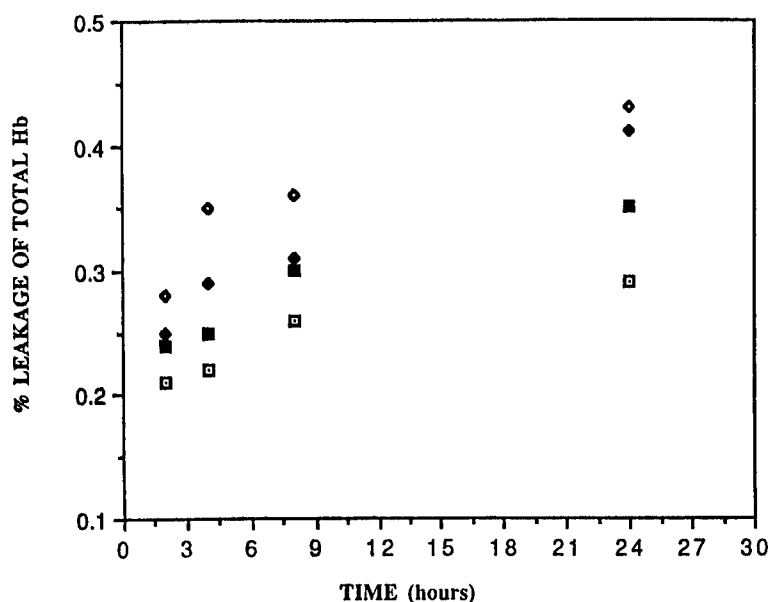
FIGURE 2.   Effect of incubation time of PI LEH in human plasma◆, in
                   7.5 wt% albumin-PBS ▣ , and PEG-PE LEH in human
                   plasma◇, and in 7.5 wt% albumin-PBS ■ on Hb leakage
                   at 37°C.

other conventional phospholipids formulations.   A possible mechanism being
developed to explain this behavior is that the PEG-PE polymer-phospholipid
component sterically stabilizes the LEH [23] like that previously reported for the
nonionic surfactant coating of colloidal particles [37].   This results in "limited
accessibility" [13], i.e because of steric stabilization, of the liposome surface to
adsorption by plasma proteins [37] such as immunoglobulins and high density
lipoproteins, which could cause vesicular breakdown [39] and opsonization
followed by RES uptake [13].   Also, it was recently shown that the binding of
phosphatidylglycerol liposomes to rat platelets was mediated by complement [17].
Recently it was found that replacement of PI with PEG-PE in LEH may provide
a mechanism for in vivo oxygen delivery with less adverse impact on host
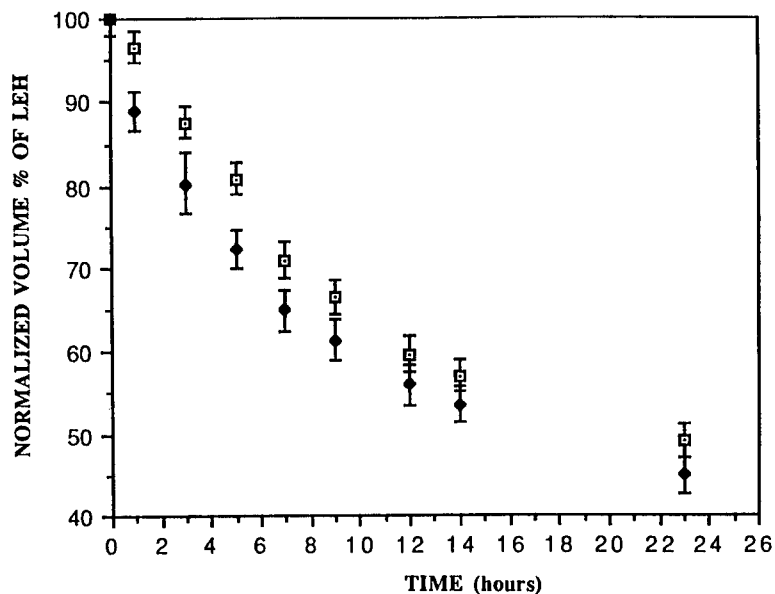
FIGURE 3.   Clearance of PI LEH ◆ and PEG-PE LEH ⊡ in rats
                following 50% isovolemic exchange transfusion.

resistance and immunity [19]. Groups of CD-1 mice were dosed with LEH suspension or albumin control solution on Day -3 or Day 1 relative to i.v. infectious challenge on Day 0 with a 20% lethal dose of *Listeria monocytogenes*. Mice dosed with LEH containing PI on Day -3 retained essentially normal immune response against *Listeria* infection. However, mice dosed with LEH containing PEG-PE had increased mortality. Mice challenged on Day 1 with LEH containing PI rapidly succumbed to *Listeria* infection, whereas mice dosed with LEH containing PEG-PE also died more rapidly than controls, but mortality rates were significantly decreased as compared to LEH containing PI-exposed mice. These results suggested that LEH containing PI clearance by the RES was extremely rapid so that mice dosed on Day -3 had already overcome the RES overload while challenge on Day 1 caused extreme immunosuppression resulting
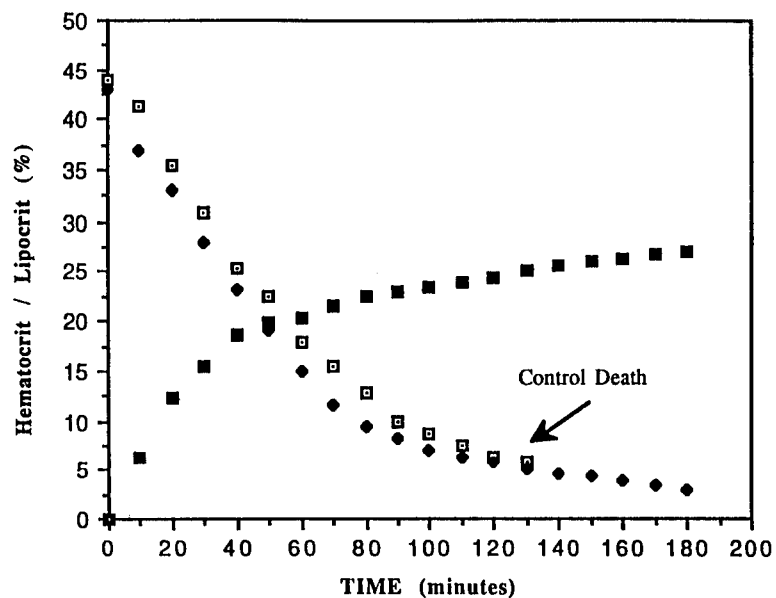
FIGURE 4A.   Hematocrit in rats during isovolemic exchange transfusion
              with 7.5 wt% albumin-PBS ☐ and with PEG-PE LEH ◆.
              The lipocrit of PEG-PE LEH ■ during the exchange is also
              shown.
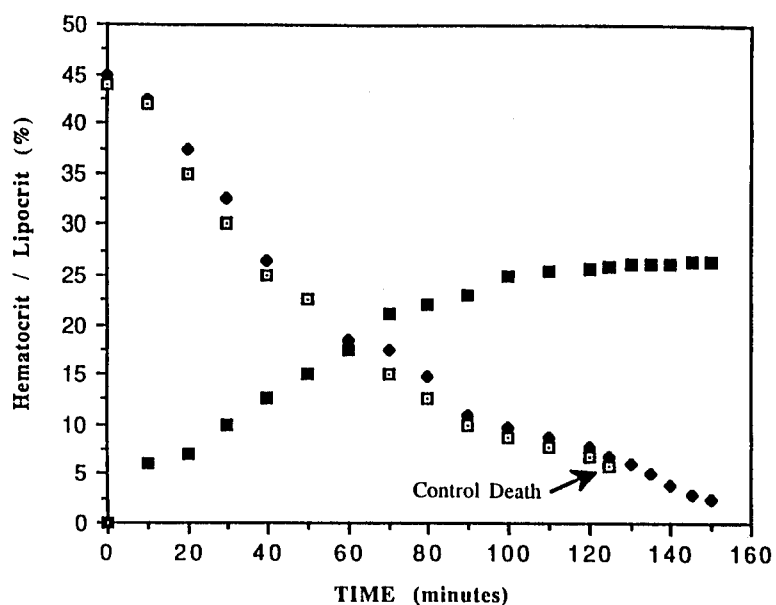
FIGURE 4B.   Hematocrit in rats during isovolemic exchange transfusion
              with 7.5 wt% albumin-PBS ☐ and with PI LEH ◆. The
              lipocrit of PI LEH ■ during the exchange is also shown.

in overwhelming *Listeria* infection and death. LEH containing PEG-PE also impaired immune defenses, but to a lesser degree, which suggested a more prolonged clearance pattern with less impairment of the phagocytic capabilities of the RES.

As shown in Figures 4a and 4b, nearly total (97%) isovolemic exchange transfusion demonstrates efficacy of PI and PEG-PE based LEH suspension samples, since administration of LEH supported life in rats whose hematocrit had been reduced to levels below 5% which are incompatible with survival when performing exchange transfusion with isotonic/isooncotic PBS containing 7.5 g% albumin. These results confirming the efficacy of PI are consistent with those found in other recent studies for terminal hematocrit obtained for control and LEH-exchanged animals [7,16,17] and show the PEG-PE liposomes are equally efficacious.

CONCLUSIONS

The finding that PEG-PE is a suitable substitute for PI in this setting improves the prospects of developing a commercially viable liposome-based blood substitute for several reasons: PEG-PE is synthetic and thus has the potential to be produced in commercial quantities at reasonable cost, PEG-PE exhibits favorable solubility properties (similar to PC), LEH containing PEG-PE are efficacious and, perhaps most importantly, recent evidence suggests that liposomes containing PEG-PE may have less adverse impact than PI on the capacity of the MPS to clear pathogens from the bloodstream [19].

ACKNOWLEDGEMENTS

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EFFECT OF NEO RED CELLS ON HEMODYNAMICS AND
BLOOD GAS TRANSPORT IN CANINE HEMORRHAGIC
SHOCK AND ITS SAFETY FOR VITAL ORGANS

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ABSTRACT

The purpose of this study was to evaluate the effects of liposome encapsulated hemoglobin named "Neo Red Cells (NRC)" on canine hemorrhagic shock model and its safety for the vital organs in a whole blood exchange model. HEMORRHAGIC SHOCK: Nine adult mongrel dogs were used. Under mechanical ventilation inhaling room air, blood was withdrawn via an artery at a rate of 40 ml / min in order to induce hemorrhagic shock (systolic pressure below 60 mm Hg) and then NRC was transfused. For each animal, three to five cycles of bloodletting and NRC transfusion were performed. After blood exchange, total peripheral resistance index (TPRI) decreased and cardiac index (CI) increased. These changes were more marked in the high exchange group (exchange rate over 88%; five animals) than in the low exchange group (less than 88%; four animals), indicating that the low viscosity NRC reduced the load on the circulatory system. The A-V difference

503

in oxygen content per 1g hemoglobin was greater after blood exchange, indicating that oxygen binding capacity of NRC is higher than that of red blood cells. WHOLE BLOOD EXCHANGE: Five beagles were used for the blood exchange. The blood was withdrawn from an artery at a rate of 15 ml / min and NRC was infused at the same time. A dog whose blood was exchanged with hydroxyethylstarch instead of NRC died within 15 hours after blood exchange. Three dogs whose blood was exchanged with NRC (exchange rate was from 82 to 90%) have been living over a year without any side effects. A dog sacrificed on the 15th postoperative day for autopsy, microscopically showed no side effects in vital organs. We conclude that NRC is more suitable than natural blood for treatment of hemorrhagic shock and safe for vital organs.

INTRODUCTION

Blood transfusion is necessary in cases of hemorrhagic shock. Recently, however, the side effects of blood transfusion are being increasingly noted, and the advantages and disadvantages of blood transfusion in light of viral infectious diseases such as hepatitis B, hepatitis C and acquired immunodeficiency syndrome (AIDS), and graft-versus-host-disease (GVHD), have been debated. Since blood transfusion is necessary for hemorrhagic shock, the development of artificial blood is seen as a means to resolve these problems. Since 1978 we [1-8] have studied the oxygen-transport capacity of perfluorochemicals, and reported the first clinically successful case in the world. Since then various improvements have been made. We [9-11] developed Neo Red Cells (NRC), which include concentrated stroma-free hemoglobin(SFH) in liposomes. In this study, the effect of transfused NRC on hemorrhagic shock and safety in whole blood exchange are evaluated.

MATERIALS AND METHODS

1. Preparation of NRC

From human concentrated erythrocyte preparations, whose recommended period of use has expired, hemoglobin is extracted and purified to obtain SFH. To improve the oxygen-transporting efficiency, inositol hexaphosphate (IHP) is added to it. Subsequently, it is mixed well with hydrogen-added yolk phosphatidylcholine, cholesterol, myristic acid, and presome, followed by liposome conjugation using a high speed agitator. In this way, NRC is prepared. To adjust the colloid osmotic pressure, hydroxyethylstarch is added, resulting in a hemoglobin concentration of 5.6 g / dl (TABLE I).

2. Features of NRC

NRC can be characterized by low viscosity and high oxygen transporting capacity.

1) Low viscosity

Capsules with a high–molecular–weight membrane usually have a low density. If highly concentrated SFH is encapsulated, the viscosity per unit hemoglobin is further reduced. When the hemoglobin concentration is 5.6 g / dl, the NRC viscosity is 2 cp, which is only about half that of SFH viscosity and about 1/3 that of whole blood at the same hemoglobin concentration [11].

2) High oxygen-transporting capacity

The affinity of hemoglobin for oxygen is regulated by 2,3-diphosphoglyceric acid (2,3-DPG). If the 2,3-DPG level is not high enough, the hemoglobin affinity for oxygen is abnormally elevated, resulting in a leftward shift of the oxygen dissociation curve and a reduction in the oxygen- transporting capacity. Thus, 2,3-DPG regulates the oxygen-transporting capacity by appropriately regulating the hemoglobin's affinity for oxygen. Like 2,3-DPG, inositol hexaphosphate is known to regulate the affinity for oxygen. If the oxygen dissociation is regulated by appropriately adding this substance, the oxygen-transporting capacity can be made greater than that of natural erythrocytes [11].

TABLE I   Preparation of NRC

hemoglobin concentration	5.6 g/dl
mean particle size	180 ± 88 nm
number of bimolecular lipid membrane	2-7 pieces
NRCct	29.6 %
methemoglobin rate	5 %
P_{50}	49.5 mmHg
viscocity	2 cp
colloid oncotic pressure electrolyte concentration	approx. 25 mmHg
Na^+	154 mEq/L
Cl^-	154 mEq/L

2. Experimental hemorrhagic shock

Nine adult mongrel dogs, weighing 10.0–13.0 kg , were used. Under intravenous anesthesia with 250 mg of ketamine sulfate, tracheal intubation was performed to mechanically control the ventilation at a respiration rate of 20 / min, a ventilation volume of 400 ml , and an inhaled oxygen concentration of 20.9% (room air). Pancuronium bromide (0.08 mg / kg) was also injected intravenously.

Hemorrhagic shock (systolic pressure below 60 mmHg) was induced by drawing blood via the right femoral artery at a rate of 40 ml / min. Immediately after induction of hemorrhagic shock, NRC was transfused at a constant rate in a volume equal to the blood lost. The same procedure was repeated 3–5 times.

Based on the blood exchange rates, the 9 animals were divided into two groups: Group I (less than 88% exchange) and Group II (88% or more exchange). Between these two groups, the following parameters were compared: blood exchange rate, volume of blood lost, amount of

NRC administered, red blood cells hemoglobin level (R−Hb) before and after blood exchange, post-exchange hemoglobin levels in NRC (NRC −Hb). Blood exchange rate was calculated, using the following equation:

Blood exchange rate(%)
$$= [\{\text{Erythrocyte Hb (g/dl) before blood exchange}$$
$$- \text{Erythrocyte Hb after exchange}\}/\text{Erythrocyte Hb}]$$
$$\times 100$$

A catheter for monitoring arterial pressure and Swan-Ganz catheter were inserted via the femoral artery and the femoral vein, respectively. Using these catheters, the following hemodynamic parameters were measured: systemic blood pressure (SBP), heart rate (HR), cardiac ortput (CO), pulmonary artery pressure (PAP), pulmonary artery wedge pressure (PWP) and right atrial pressure(RAP). Based on these measurements, the following parameters were calculated: cardiac index(CI), total peripheral vascular resistance index (TPRI).

Before and after blood exchange, arterial and venous blood were examined for PO_2, PCO_2, pH, HCO_3, BE, NRCcrit, percentage of degradated Hb and oxygen saturation of NRC (arterial and venous). From these measurments, the following parameters were calculated: NRC's oxygen content (arterial and venous), the differnce: between arterial and venous NRC oxygen contents (A−V difference). All animals were sacrificed for autopsy after blood exchongo.

3. Experimental whole blood exchange

Five beagles weighing 7.4 to 10.8 kg were used for this experiment. Catheters were inserted in the femoral artery and vein under intravenous anesthesia and connected to peristaltic pumps. Exsanguination and NRC transfusion were performed simultaneously at a rate of 15 ml/min. The Hb concentration of NRC was 6.0g/dl. Hydroxyethylstarch (HES) 5g/dl was used as the external solution. Dog 1 served as the control: ie., its blood was exchanged with HES alone. In dogs 2, 3 and 4, the blood was exchanged with HES until the hematocrit level became

about 20%; then the blood was exchanged with NRC. In addition 500 ml of NRC was administered on the day after blood exchange. In dog 5, the blood was exchanged with NRC alone. The pH was corrected with bicarbonate as needed immediately after blood exchange but not subsequently. Hematocrit (Ht), blood sugar, and lactic acid were determined by blood gas analysis before and after blood exchange. Dog 4 was sacrificed 15 days after blood exchange for histological investigation.

All values were expressed in mean ± SD. Significance of differences was tested by Student's t–test. $p < 0.05$ was regarded as significant.

RESULTS

1. Experimental hemorrhagic shock

TABLE II shows rate of blood exchange and hemoglobin concentration. There was significant difference in hemoglobin concentration in red blood cells (R–Hb) between groups I and II after blood exchange.

Along with increase of blood exchange, blood pressure decreased and the heart rate increased in Groups I and II. The pulmonary arterial pressure, pulmonary wedge pressure and right atrial pressure decreased slightly and pulmonary arterial resisaence index decreased after blood exchange. Cardiacindex (CI) increased in both groups after blood exchange. The percent CI after blood exchange, relative to the value before exchange, differed significantly between Groups I and II. In contrast to CI, the total peripheral vascular resistance index (TPRI) decreased markedly after blood exchange in Groups I and II. However, the difference in TPRI value between the two groups was not significant (FIGURE I). FIGURE I also shows the difference in oxygen content per 1g hemoglobin between arterial and venous blood. Before blood exchange, this parameter was 0.20 ± 0.08 ml / dl /g in Group I and 0.13 ± 0.04 ml / dl /g in Group II. After blood exchange

TABLE Ⅱ Blood exchange rates and hemoglobin concentrations
in hemorrhagic shock model.

	Group Ⅰ	Group Ⅱ	
Naumber of dogs	4	5	—
Exchange rate (%)	70.3 ± 9.4	90.3 ± 3.3	p < 0.05
Blood loss (ml)	920 ± 310	1315 ± 373	NS
NRC transfusion (ml)	1002 ± 318	1455 ± 432	NS
R − Hb (g/dl) (before exchange)	14.6 ± 3.1	14.7 ± 2.6	NS
R − Hb (g/dl) (after exchange)	4.2 ± 1.2	1.4 ± 0.6	p < 0.05
NRC − Hb (g/dl)	3.4 ± 0.6	4.0 ± 1.2	NS

with NRC, it was $0.41 ± 0.18$ ml/dl/g in Group Ⅰ and $0.55 ± 0.03$ ml/dl/g
in Group Ⅱ. Before and after blood exchange, this parameter tended
to be slightly higher in Group Ⅱ than Group Ⅰ. This analysis disclosed
a nearly two fold increase of arterial-venous oxegen difference in
Group Ⅰ and a neary four fold increase of the difference in Group Ⅱ
after blood exchange compared to before blood exchange.

2. Experimental whole blood exchange.

TABLE Ⅲ shows body weight, dose of HES or NRC, amount of
exsanguination, rate of blood exchange, and results of blood exchange.
The rate of blood exchange was 82−90%. Dog 1, whose blood was ex-
changed with HES alone, died 15 hours after exchange, while dog 2
and 3, whose blood was exchanged with HES and NRC, and dog 5, whose
blood was exchanged with NRC, are alive for 21 months, 20 months and
19 months, respectively, following exchange. Dog 4 was sacrificad for
histological examination.

TPRI (Δ %)

(%)
200

100

0

　　#1　　#2

CI (Δ %)

(%)
200

p<0.05

100

0

　　#1　　#2

(Cₐ−Cv)/Hb

(ml/dl/g)
1.00

0.80

0.60

0.40

0.20

0

　#1　　#2
Red Cells
(before exchange)

　#1　　#2
NRC
(after exchange)
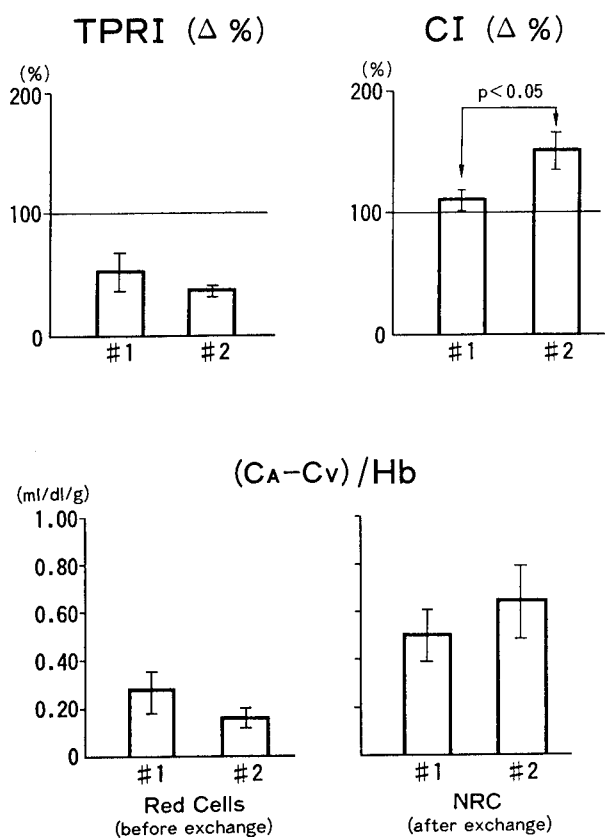
FIGURE I. Percent changes in cardiac index (CI) and total peripheral
vascular resistance index (TPRI) after blood exchange, and
A-V difference in oxygen content per 1g hemoglobin before
(red cells) and after (NRC) blood exchange.

The erythrocyte count was markedly decreased immediately after
blood exchange in dogs 2, 3, 4 and 5, but about 2 weeks later it returned
to nearly the preoperative value.

FIGURE II shows changes in PaO_2, PvO_2, $PaCO_2$ and pH in dogs 2,
3, 4 and 5. Each of the dogs showed a $PaCO_2$ of about 100mm Hg and
PvO_2 of about 40 mm Hg, showing almost no change. There was no
tendency toward elevation of $PaCO_2$. Although pH decreased imme-

TABLE Ⅲ Conditions and outcome of the whole blood exchange studies.

DOG	BODY WEIGHT (kg)	INFUSION (ml)		BLOOD LOSS (ml)	EXCHANGE RATE (%)	RESULT
1	7.4	1090	HES	990	84	Died. (15 hr)
2	7.9	500 980	HES NRC	1380	84	Alive (21 mo)
3	8.0	700 970	HES HSE	1600	82	Alive (20 mo)
4	8.0	700 970	HES NRC	1600	82	Sacrificed (15 day)
5	10.8	2000	NRC	1900	90	Alive (19 mo)

diately after the exchange in some of the animals, thereafter the level showed almost no change.

FIGURE Ⅲ shows changes in the blood sugar and lactic acid levels in dogs 2, 3, 4 and 5. One showed a slightly high level of blood sugar, but the lactic acid level was unchanged in these 4 dogs.

Dog 4 was sacrificed 15 days after blood exchange and was histologically examined (heart, lung, liver, kidney and spleen). Although macrophages phagocytosing NRC were present in the lung and spleen, NRC were not observed in Kupffer cells of the liver, which might have phagocytosed NRC. There were no histologically abnormal findings in the heart or kidnies.

DISCUSSION

Until now, various blood substitutes have been studied. Artificial blood can be roughly divided into three types, depending on which of

USUBA ET AL.
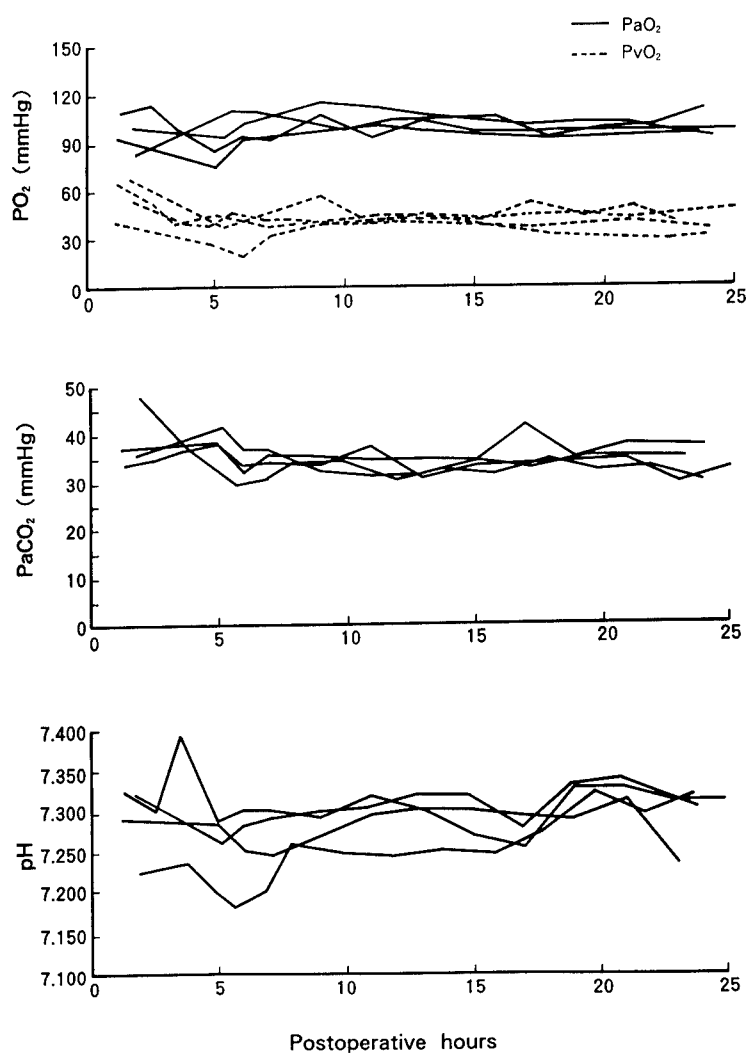
FIGURE II. Changes in blood gas analysis after blood exchange with
           NRC in four dogs.
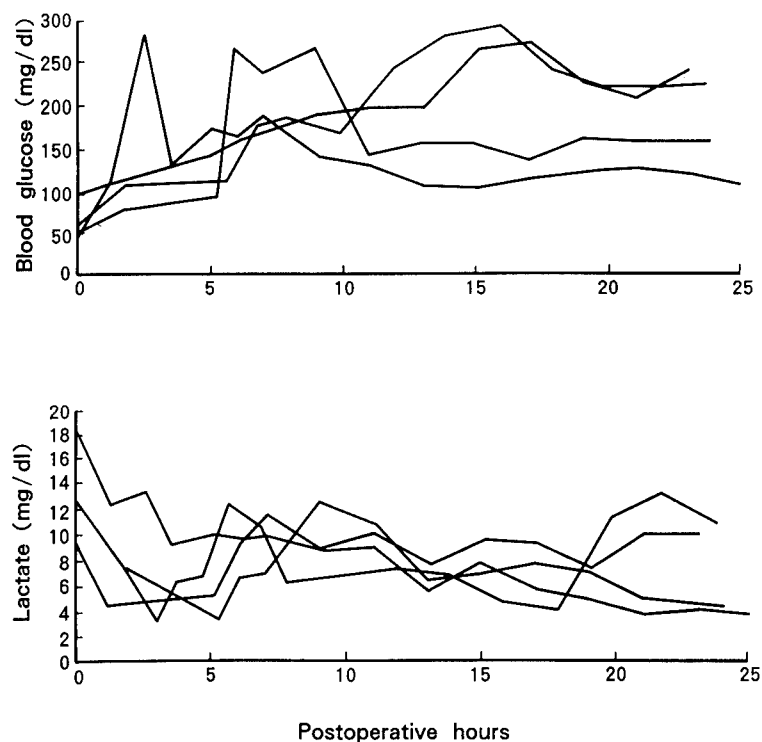
FIGURE III. Changes in blood glucose and lactate after blood exchange
          with NRC in four dogs.

the following three are used for oxygen transportation: (1) high—
molecular—weight metal complex, (2) perfluorochemicals, and (3) hemo-
globin, of these three oxygin carriers, high—molecular—weight metal
complexes are rarely used at present because of the problem of rever-
sible binding to oxygen. We first examined the usefulness of perfluoro-
chemicals in patients with hemorrhagic shock.   However, perfluoro-
chemicals were found not only to have low oxygen transporting ca-
pacity but also to have a tendency to accumulate in the body [5].

USUBA ET AL.

Subsequently, hemoglobin-based artificial blood began to play a principal role in artificial blood, taking the place of fluorocarbon-based artificial blood. We developed NRC by encapsulating stroma-free hemoglobin into a liposome microcapsule.

Circulatory changes will be discussed first. Blood pressure was decreased after blood exchange in groups I and II, and the heart rate increased. Although there was no significant difference in TPRI, this parameter decreased in groups I and II. Assuming the previous level to be 100%, TPRI decreased by half to 52.2% in group I and decreased to 35.3%, in group II The decrease in TPRI was attributed to the fact that NRC easily passed through peripheral blood vessels constricted by shock since the viscosity of NRC was 2 cp, i.e., 1/3 that of whole blood, and the particle size of NRC was only 1/40 that of erythrocytes. The decrease in TPRI might have increased CO and CI and reduced the left cardiac burden, leading to decreases in blood pressure.

Because it was difficult to establish a control, the A–V difference (equivalent to 1 g of Hb) NRC and erythrocytes before blood exchange was compared. As a control for the present experiment, it seems more reasonable to use lavaged erythrocytes than to return whole blood mixed with platelets and leukocytes, thus avoiding the use of anticoagulants and the presence of chemical mediators. However, since simultaneous lavage of erythrocytes is difficult and the individual differences were large because mongrel dogs were used, erythrocytes before blood exchange were compared with NRC after exchange in the same dog and under the same conditions as those before exchange. The A–V difference increased about two-fold in group I and about 4-fold in group II.

These results suggest that NRC bind oxygen at a rate 2 to 4 times greater than natural erythroytes and oxygen transport increases with the rate of exchange. This was considered to have occurred by the following mechanism: as a result of increased oxygen affinity due to the addition of IHP, the oxygen dissociation curve of NRC becomes more similar to a straight line than does that of natural erythrocytes

[11], and it become possible to bind a larger amount of oxygen than would be possible with natural erythrocytes.

In the experiment of whole blood exchange, the safety of NRC was investigated in animals that were not sacrificed. Although the dog whose blood was exchanged with HES alone died 15 hours later, 4 dogs whose blood was exchanged with NRC + HES or NRC survived. Excluding the one that was sacrificed the remaining 3 are alive without side effects after more than one year.

NRC in blood vessels were soon phagocytosed by reticuloendothelial cells and removed as foreign bodies. Therefore, the recovery of auto-erythrocytes is an issue. Three of the 4 surviving dogs, whose blood was preliminarily diluted with HES, required additinal administration of 500 ml of NRC on the day after exchange, whereas the dog whose blood was exchanged with NRC alone, did not require this supplementation. Moreover, the Hct level returned to the preoperative level 2 weeks after exchange in the dog.

As NRC disappears from the blood, oxygen-transfer capacity becomes an issue. According to follow-up for 25 hours after the exchange, blood gas analysis revealed no specific problem. Although the blood sugar level was slightly high in some cases, the lactic acid level was not problematic. Oxygen was considered to have been adequately supplied to the periphery.

The histologic findings showed NRC phagocytosed by reticulo-endothelial cells. In dog 4, which was sacrificed 15 days after the operation, only a few phagocytosed NRC were observed in the spleen and lung, and there were no NRC in Kupffer cells of the liver. This suggested that NRC were removed rapidly.

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II. POSTERS

ENHANCED OXIDATION OF BIS(3,5-DIBROMOSALICYL) FUMARATE
α–α CROSS LINKED HEMOGLOBIN BY FREE RADICALS
GENERATED BY XANTHINE/XANTHINE OXIDASE

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ABSTRACT

The xanthine/xanthine oxidase reaction produces reproducible amounts of oxygen-derived free radicals that oxidize human oxyhemoglobin (Hb). We monitored the kinetics of the oxidation of stripped Hb (sHb), purified HbA_0 and α–α cross-linked Hb (HbXL99α) at [Hb] in the 5 to 150 μM (heme) range. For increasing [Hb], the oxidation halftime ($t_{1/2}$) increased for all Hbs, but $t_{1/2}$ was always less for HbXL99α than for HbA_0 and sHb. Such feature was attributed to the lower affinity for O_2 of HbXL99α and may represent a serious problem for use of this Hb as blood substitute.

INTRODUCTION

The cytotoxicity of free plasma hemoglobin (Hb) involves free radicals [1,2]. Hb is known to generate free radicals during autooxidation [3,4] and to increase through the Fenton reaction the peroxidation of fatty acids initiated by free radicals generated by external sources [5-7]. Here, we focus on Hb as *target* of oxygen-derived free radicals (ODFR) generated by xanthine (XAN) and xanthine oxidase (XO). The main

517

features of this approach are: 1) The amount of ODFR is modulated and reproducible because they are produced stoichiometrically by an enzymatic system; 2) The primary target of ODFR is distinguished from secondary targets such as peroxidation of lipids; 3) The time course of this reaction is faster than that of Hb autooxidation; 4) The effects of ODFR are easily monitored with spectrophotometric methods.

We characterized this model monitoring the spectral changes of HbO_2 when mixed with XAN/XO assuming the mechanism drawn in Fig.1 (unbalanced equations). We show that ODFR oxidize HbO_2 in a concentration-dependent fashion with an action likely directed towards the heme. The Hb derivative cross-linked between the α chains by bis(3,5-dibromosalicyl) fumarate ($HbXL99\alpha$) appeared more susceptible than stripped Hb (sHb) and HbA_0 to the oxidation by ODFR perhaps for its lower O_2 affinity.

MATERIALS AND METHODS

Materials. The medium contained 50 mM K_2HPO_4, 1 mM ethylenediaminetetraacetate (EDTA), 0.5 mM XAN, 20 mM KCN, pH 7.3 at 37_C. Dimethylthiourea (DMTU, Aldrich), XO (20 U/mL, cow milk), superoxide dismutase (SOD, 5,000 U/mg, bovine erythrocytes), catalase (260,000 U/mL, beef liver), and cytochrome c (Boehringer Biochemia) were used without further treatment.

We prepared sHb from freshly drawn blood of non-smoker donors (HbCO<2%) [8]. Total [Hb] was determined by the met-cyanide method [9]. MetHb was obtained mixing HbO_2 with 20% molar excess solid $K_3Fe(CN)_6$ for 15 min at room temperature [8]. HbA_0 was purified by anion exchange high-performance liquid chromatography [10]. $HbXL99\alpha$ was obtained reacting HbA_0 with 3,5-bis-dibromosalicyl-fumarate [11].

Methods. Visible and UV spectra were obtained with a DU-70 (Beckman Instruments Inc., Fullerton, CA). Kinetics were recorded at 37 C by a DW-2a (American Instrumentation Co., Silver Spring, MD) dual wavelength spectrophotometer operating at 577-600 nm (3 nm slit opening). The 1-cm path length quartz cuvette was filled with 1 mL of air-saturated buffer ([O_2]_0.2 mM [12]) and appropriate amounts of HbO_2 were added to it. The reaction was started by adding 100 mU/mL XO to
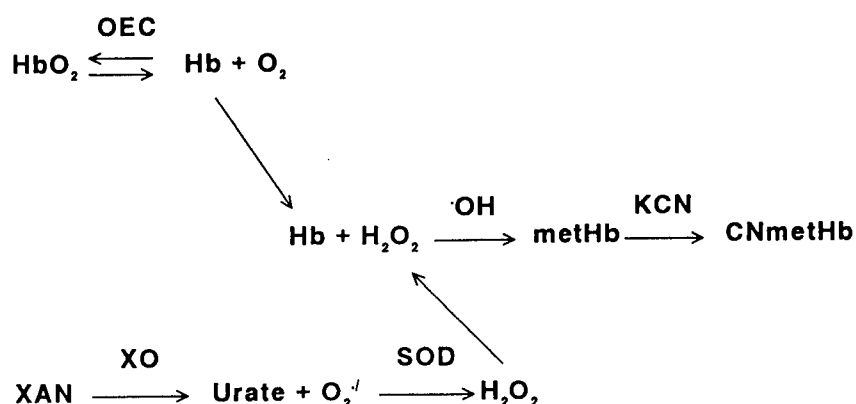
FIGURE 1. Scheme of the mechanisms assumed in this work (unbalanced reactions). Abbreviations: OEC, oxygen equilibrium curve; SOD, superoxide dismutase; XO, xanthine oxidase.

the mixture. The rate of $O_2 \cdot^-$ production by XAN/XO was measured mixing 100 μM cytochrome c ($E_{550\ nm}$=24.3 mM^{-1} cm^{-1} in the reduced form) with 100 mU/mL XO and 0.5 mM XAN [13]. The Hb-O_2 affinity was measured at pH 7.4, 37°C (Hemox oxygen equilibrium curve analyzer).

RESULTS

Generation of ODFRs and Hb spectra. Under the selected conditions, $O_2 \cdot^-$ was generated steadily at 0.045 mM/min for 2 min without initial lag phases. The oxidation of HbO$_2$ (all types) by 0.5 mM XAN or urate in the absence of XO was <0.25 μM/min (0.8%/min) and <0.6 μM/min (2%/min), respectively. The oxidation rate was not limited by shortage of XAN nor O$_2$, which was regenerated during the reaction (Fig.1). KCN was added to the medium to convert metHb to CNmetHb.

Spectra analysis in the visible and UV regions (not shown) indicated that HbO$_2$ was oxidized to CNmetHb. The absorbance change at 700 nm, index of aspecific protein denaturation [14], was <0.003 absorbance units/min under all conditions.

Kinetics. The oxidation of 50 μM/L sHb (Fig.2) was typically sigmoidal with maximal rate (V_{max})=22.7±2.1 μM/min (mean±SD, n=10).
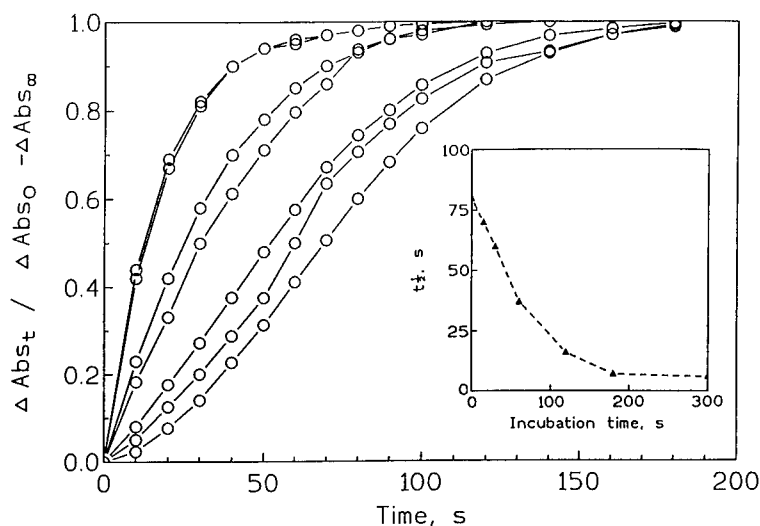
FIGURE 2. Kinetics of the oxidation of 50 μM stripped HbO_2 by pre-incubated mixtures of XAN/XO (from right to left, 0, 15, 30, 60, 120, 180 and 300 s). Each point represents the mean of 4 to 6 experiments (2% average S.D., not shown for clarity). $\Delta Abs = Abs_{577} - Abs_{600}$. Insert: Half-time ($t_{1/2}$) of the reaction vs the corresponding incubation time.

In the presence of CO, V_{max} was 0.7 ± 0.3 μM/min (n=5, p<0.0001). When sHb was added to pre-incubated XAN/XO (0 to 300 s) the sigmoidal shape disappeared and the oxidation halftime ($t_{1/2}$) decreased as function of the incubation time (Fig.2).

The oxidation of sHb was indistinguishable from that of HbA_0. Fig.3 shows the increase of $t_{1/2}$ at increasing [Hb] in the 5 to 150 μM range. At all concentrations, $t_{1/2}$ for $HbXL99\alpha$ was significantly less than for HbA_0.

In the presence of 50 mM DMTU, scavenger of $\cdot OH$, V_{max} reduced to 15.1 ± 1.2 μM/min (n=6, p<0.0001). In the presence of 2000 U/ml SOD, scavenger of O_2^{-}, V_{max} increased by 7-9% (p=NS). The effect of catalase, which quickly decomposes H_2O_2, was not tested for the inhibition of this enzyme by KCN, but experiments in the absence of KCN demonstrated that catalase prevents the oxidation of HbO_2 (not shown).
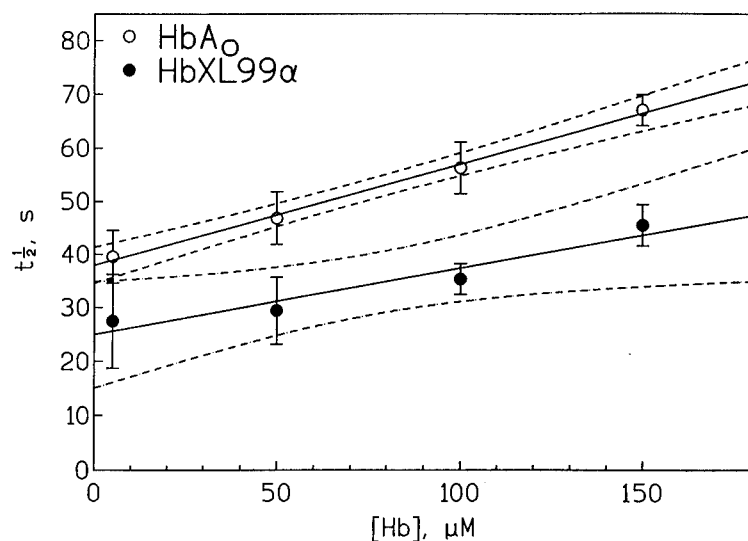
FIGURE 3. Halftime ($t_{1/2}$) of the oxidation of HbXL99α and HbA$_0$ (virtually the same as stripped Hb) as function of [Hb]. Dashed lines are the 95% confidence limits. The differences were significant at the p=0.03, 0.002, 0.0005 and 0.0005 levels (Student's t-test for unpaired data) at the four [Hb]'s.

The P_{50}'s of stripped Hb, HbA$_0$ and HbXL99α were 10.0, 11.5 and 30.0 mmHg, respectively, pH 7.4.

DISCUSSION

The XAN/XO system generates reproducible and accurate amounts of ODFRs that oxidize HbO$_2$ by specific mechanisms which are faster than HbO$_2$ autooxidation [6]. The oxidation rate depended on [Hb] for all Hb's, but HbXL99α was always oxidized faster than sHb and HbA$_0$.

Kinetic data fit a model where the first product of XAN/XO (O$_2$·⁻) is unable to oxidize HbO$_2$, but slowly dismutes to H$_2$O$_2$ which is a strong oxidant. Several observations support this view: 1) When HbO$_2$ was added to pre-incubated mixtures of XAN/XO, the sigmoidal shape of the oxidation curve disappeared and the oxidation was faster; 2) SOD slightly

SAMAJA, MOTTERLINI, AND ROVIDA

increased the oxidation rate presumably accelerating the formation of H_2O_2 [15-17]; 3) Catalase prevented the oxidation; 4) DMTU slowed significantly the oxidation rate scavenging the $\cdot$OH radical.

The dependence of the oxidation rate on [Hb] could be related to the Hb tetramer splitting into dimers and to different reactivities of tetramers and dimers. For a dissociation constant of 0.001 mM [18], the tetrameric form accounts for 92.2% and 64.2% of total Hb at [Hb]=150 and 5 μM, respectively. However, the presence of $\alpha-\alpha$ covalent linkage in HbXL99α should have prevented splitting in this Hb, but nevertheless the kinetics of HbXL99α depended on [Hb] in the same way as HbA_0 and sHb, indicating that it is unlikely that the Hb tetramers and dimers have different reactivities in this model.

The heme appeared the elective site of action of ODFR because the oxidation was nearly abolished when O_2 was replaced by CO, consistently with the slow rate of CO dissociation from HbCO [19]. The faster oxidation of HbXL99α with respect to that of HbA_0 and sHb may thus be explained hypothesizing that the overall reaction is limited by the release of O_2 from HbO_2 reflecting different Hb-O_2 affinities of the various tested Hbs.

These data are in essential agreement with the faster autooxidation rate of HbXL99α with respect to HbXL82β and HbA [20]. However, we suggest that it is not only during storage that HbXL99α must be protected from oxidation, but also when infused into a patient, because this Hb is particularly susceptible to oxidation by circulating oxidative factors, especially those arising from reperfusion of ischemic tissues.

ACKNOWLEDGEMENTS

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CYTOKINES AND PAF RELEASE FROM HUMAN MONOCYTES AND MACROPHAGES: EFFECT OF HEMOGLOBIN AND CONTAMINANTS

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ABSTRACT

Monocytes [M] were isolated from venous blood of healthy volunteers and activated macrophage-leukocytes (MØ-L) were obtained from peritoneal fluid of patients with mild endometriosis. The M were incubated with pyrogen free CELLGRO culture medium [Control], and with 0.2 mM of [A] unmodified bovine hemoglobin (UHb), [B] Hb crosslinked to form polymers with M.W.<400 kDa (LMWHb), [C] Hb crosslinked to form large polymers (<1,020 kDa) (HMWHb), and MØ-L additionally with [D] UHb contaminated with endotoxin (Hb+E) (2.5 EU/mL), and [E] UHb contaminated with phospholipids (Hb+PLs). The MØ-L medium of incubation was tested for TNFα, IL-1α, IL-6, GM-CSF and PAF after 6 and 24 hours, but M for TNFα and GM-CSF at 12, 24 and 36 hours. MØ-L were found more responsive than M colonies. The strongest reaction of MØ-L was to Hb+E, which produced levels of cytokines and PAF higher than Controls (p<0.001). Hb+PLs induced smaller increases of TNF and IL-6, and a decrease in the levels of IL-1 and GM-CSF. However, the release of PAF was much greater with this Hb than with Hb+E. UHb caused an increase in TNF, as compared to control (p<0.01). LMWHb generated a similar increase in TNF, but also a decrease in IL-1. Both polymerized Hb forms inhibited expression of GM-CSF. HMWHb induced high levels of TNF, IL-1 and PAF. UHb, LMWHb and HMWHb significantly increase levels of TNF in M cultures after 36 hours of incubation.

INTRODUCTION

Recent scientific reviews have emphasized that polypeptide and lipid mediators released by mononuclear cells form a network of intra- and intercellular signals that orchestrate inflammatory and immunological events and influence the function of red marrow cells in their hematopoietic activities [1-3]. The stimulation of monocyte/macrophage-leukocytes (M/MØ-L) with a variety of agents including lipopolysaccharide, triggers the expression of cytokines, and produces arachidonic acid metabolites and platelet activating factor (PAF) [4, 5]. These polypeptide and lipid mediators induce many immuno-inflammatory pathological processes, such as those observed in septic shock [6].

Previous work from our laboratory has shown that impure hemoglobin (Hb) may be involved in M/MØ inflammatory reactions by releasing 6-keto $PGF_{1\alpha}$, TxB_2, H_2O_2 and lipid peroxides, and the degree of these responses is significantly increased by endotoxin, lipid impurities and Hb high molecular weight polymers [7].

The purpose of the present study was to evaluate whether different Hb solutions, and bacterial or lipid contaminants associated with Hb may directly stimulate the human M/MØ-L system to express cytokines and release of PAF.

MATERIALS AND METHODS

Hemoglobin (Hb) preparation and characterization: Pure unmodified bovine Hb (UHb) and chemically stabilized low molecular weight (M.W.) polymerized Hb (LMWHb) were prepared according to earlier developed methods [8]. High M.W. Hb (HMWHb) was crosslinked intermolecularly with glutaraldehyde [9]. Hb contaminated with nonoxidized phospholipids (Hb+PLs) was produced by the same methods described for pure UHb, however the procedure for chemical purification of PLs was not included. Hb contaminated with endotoxin (Hb+E) was prepared by adding 2.5 EU/mL of E.Coli 0111:B4 Endotoxin (Whittaker Bioproducts, Inc., Walkersville, MA). Hb solutions dialyzed against Normosol®-R at a final concentration of 1.5 mM were stored at -90°C. Purity and physical-chemical properties of Hb were characterized by earlier described quality control methods [10, 11].

Cell cultures and experimental procedures: All chemicals and culture media used in these experiments were pyrogen-free, prepared under sterile conditions.

I. MONOCYTES [M] were separated from human venous blood by using NYCODENZ® density gradient media (Nycomed AS, Oslo, Norway) according to Bøyum [12]. A monolayer of M (10^6 cells/mL/well) were incubated in 5% CO_2 at 37°C for 36 hours with DMEM medium supplemented with 5% FCS, 100 I.U./mL/100 µg/mL penicillin/ streptomycin (Mediatech, Washington, DC) (Control), and 0.2 mM of [A] UHb, [B] LMWHb (M.W.<400 kDa), [C] HMWHb (<1,020 kDa). Media were tested at 12, 24 and 36 hours for TNFα and GM-CSF. After 36 hours electron microscopic (EM) studies were performed.

II. HUMAN PERITONEAL MACROPHAGE-LEUKOCYTES [MØ-L] obtained from peritoneal fluid of women with mild endometriosis were resuspended in MEM supplemented with 5% FCS, 100 I.U./mL/100 µg/mL penicillin/streptomycin (Mediatech, Washington, DC) and cultured in Multiwell-tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ). After 12 hours, separation of nonadherent cells was performed. The MØ-L were cultured as an adherent monolayer of 0.5 x 10^6 cells/1.5 cm^2 well for 24 hours with the same solutions as M colonies [A], [B], [C], and additionally with: [D] Hb+E (2.5 EU/mL), and [E] Hb+PLs. The media of incubations were tested at 6 and 24 hours for IL-1α by RIA, and TNFα, IL-6, GM-CSF by ELISA methods (Genzyme Co., Boston, MA), and PAF by RIA (Du Pont NEN Research Products, Boston, MA), as well as EM morphological studies.

Statistical analysis: The cytokine levels in the medium of the control and Hb- treated M (n=6) and MØ-L (n=9) were compared by the Students *t*-test. Analysis was performed by using the StatWorks™ statistical package (Cricket Software, Inc., Philadelphia, PA).

RESULTS

The Hb solutions used in these experiments are characterized in **TABLE I**. Examination by overloaded isoelectric focusing and thin layer chromatography methods demonstrated the elimination of all non-Hb proteins, peptides and lipids from the UHb. The characteristics of LMWHb were described earlier [8, 13]. The chemical modification procedure with a novel crosslinking agent introduced specific changes on the surface of the Hb molecule and resulted in formation of biopolymers with M.W.<400 kDa. The distribution of polymers in HMWHb shows 41% with M.W. 408-1,020 kDa, 40%; 136-340 kDa, and 19%; 68 kDa Hb tetramers. Reaction with glutaraldehyde resulted in higher autoxidation rate (metHb level 7.5%), and higher oxygen affinity (P_{50}: 20 mmHg).

TABLE I. *Characteristics of hemoglobin solutions.*

	UHb	LMWHb	HMWHb	Hb+PLs	Hb+E
Hemoglobin, mM	1.0	1.0	1.0	1.0	1.0
Met-Hb (% of Hb)	1.5	2.7	7.5	2.8	2.7
pH, units	7.38	7.40	7.38	7.36	7.41
Osmolarity, mOsm/L	295	298	299	296	295
P_{50}, mmHg	26	23	20	25	26
Chloride, mEq/L	98	99	98	99	97
Endotoxin, EU/mL	0.08	0.12	0.12	0.09	2.5
Total phospholipids, mg/dL	Absent	Absent	Absent	4.5	Absent
Phospholipids, TLC	Absent	Absent	Absent	PE,PS,PI	Absent
Non-Hb proteins/peptides (IEF)	Absent	Absent	Absent	Trace	Absent
Mol.Wt., kDa (SEC HPLC)	68	<400	<1020	68	68

The M colonies were found less sensitive than peritoneal MØ-L (**FIG.1, FIG.2**). The highest concentration of TNF and GM-CSF was observed after 36 hours of incubation. The release of TNF from M was influenced by UHb, LMWHb, and HMWHb. Increased levels of TNF were found in M colonies incubated with UHb ($p<0.05$), LMWHb ($p<0.05$), and HMWHb ($p<0.01$). The GM-CSF expression remained low during 36 hours of observation. The UHb did not introduce significant changes in GM-CSF concentration. Incubation with LMWHb, and HMWHb resulted in inhibition of GM-CSF release ($p<0.05$).

The expression of cytokines by peritoneal MØ-L was the highest after 24 hours of incubation (**FIG.2**). The strongest reaction of these cells was to Hb+E. The level of TNF, IL-1, IL-6 and GM-CSF was significantly higher ($p<0.001$) than controls: 348, 37, 427, and 28 fold increase respectively. The Hb+PLs produced smaller increases of TNF and IL-6 (1.5 and 2.4 fold respectively), and a decrease in the levels of IL-1 ($p<0.01$) and GM-CSF ($p<0.001$). UHb caused a small increase in TNF, IL-1, and IL-6 expression. No significant changes were observed after 6 hours of incubation, however at 24 hours the levels of these cytokines were higher than control; 43% ($p<0.01$), 27% ($p<0.1$), and 28% ($p<0.05$) respectively, while the release of GM-CSF was lower ($p<0.001$). LMWHb caused a similar increase in TNF (285.4±54.3 pg/mL), but also a significant decrease (at 6 hours intervals) in IL-1 ($p<0.05$), and IL-6 ($p<0.05$), as compared to the control. Observed changes in IL-1 and IL-6 were less extensive after a 24 hour incubation, however, the levels of these cytokines remained much lower, as compared to UHb. On the contrary, HMWHb increased TNF (317.5±7.3 pg/mL) and IL-1 (177.5±13.2 pg/mL). Expression of IL-6 was not significantly influenced by HMWHb. Both polymerized Hb forms inhibited production of GM-CSF.

The release of PAF was affected by Hb solutions and contaminants (**FIG.3**). The highest increase in the PAF level was caused by Hb+PLs (0.77±0.25 ng/mL, $p<0.001$) and by Hb+E (0.62±0.48 ng/mL, $p<0.01$). The UHb and LMWHb did not cause a significant increase in PAF when compared to controls (0.275±0.12 ng/mL vs 0.353±0.10 ng/mL vs 0.234±0.09 ng/mL respectively). The HMWHb caused overproduction of PAF by MØ-L. After 24 hours levels of PAF were similar to that with Hb+E (0.532±0.15 ng/mL, $p<0.01$).
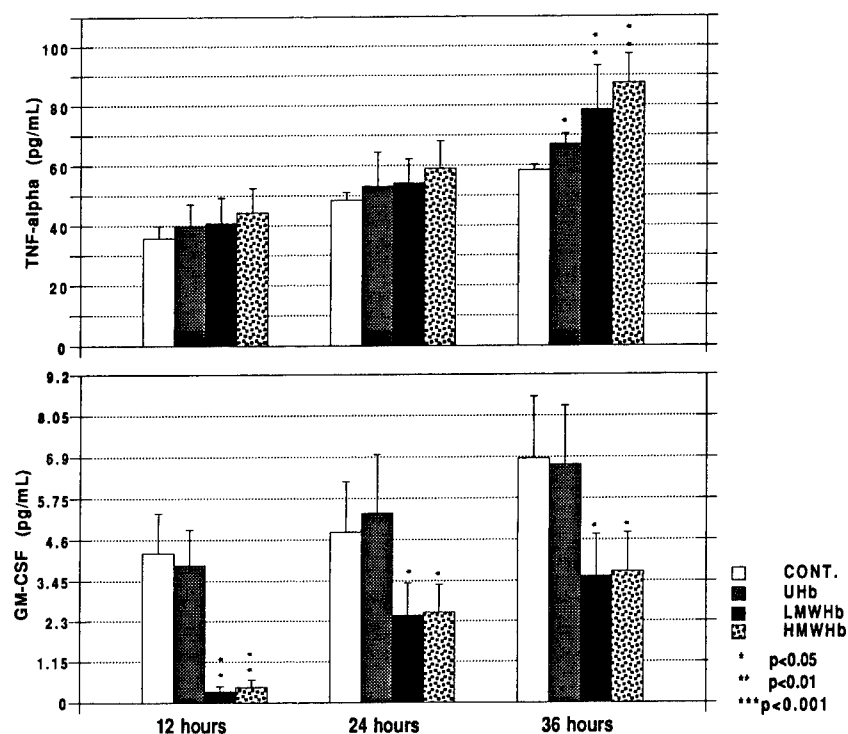
FIGURE 1. TNFα and GM-CSF release from human monocyte [M] in response to various Hb solutions. Significant differences between control and experimental groups; *** p<0.001, ** p<0.01, * p<0.05.

The EM studies (**FIG.4**) of control MØ showed the presence of numerous cytoplasmic finger-like processes and pinocytotic vacuoles. After incubation with UHb no characteristic morphological changes were observed. The LMWHb caused some MØ stimulation, indicated by the presence of empty vacuoles. The contact of MØ with HMWHb caused its hyperstimulation. Injured cells had granular cytoplasm, with intact cell membranes, large numbers of empty vacuoles and destruction of nucleus. The Hb+PLs initiated formation of MØ aggregates, loss of cytoplasmic processes and formation of empty vacuoles. Many of the dead MØ, which formed granular masses, with loss of integrity of cell membranes and disintegration of cytoplasmic structures, were observed after incubation with Hb+E.

The EM examination of M colonies revealed less severe changes after incubation with HMWHb, however a number of empty vacuoles and changes in integrity of membranes were found. The UHb and LMWHb did not introduce characteristic morphological changes.
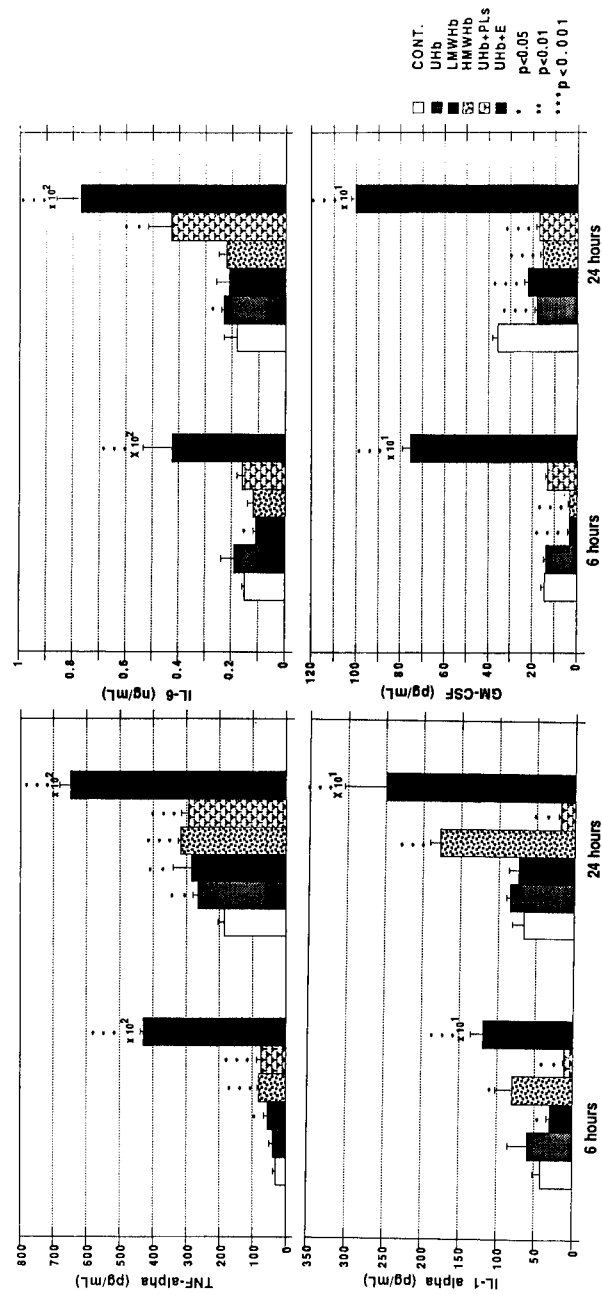
FIGURE 2. TNFα, IL-1α, IL-6 and GM-CSF expression from human peritoneal macrophage/leukocytes [MØ-L] during incubation with different Hb solutions. Significant differences; *** p<0.001, ** p<0.01, * p<0.05.

SIMONI ET AL.
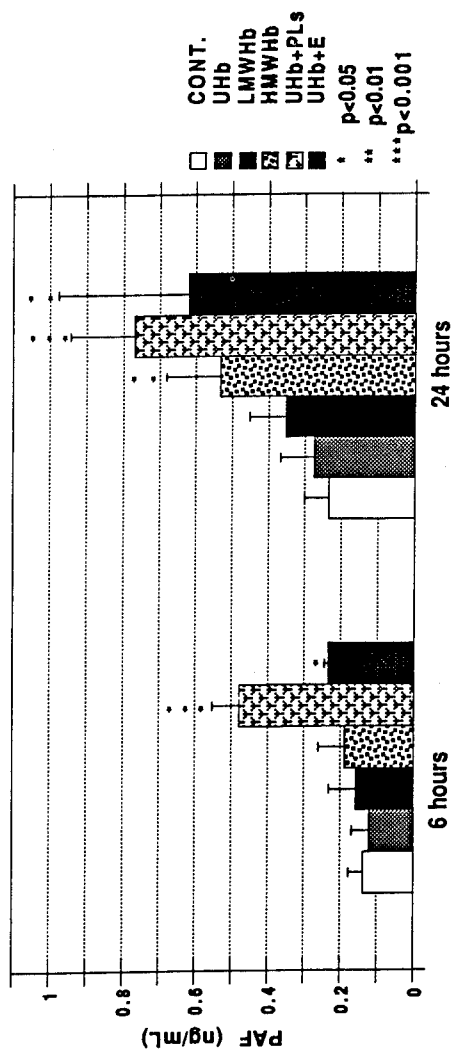
FIGURE 3. Platelet activating factor (PAF) production of human peritoneal macrophage/leukocytes [MØ-L] stimulated with different Hb solutions. Significant differences; ***p<0.001, ** p<0.01, * p<0.05.
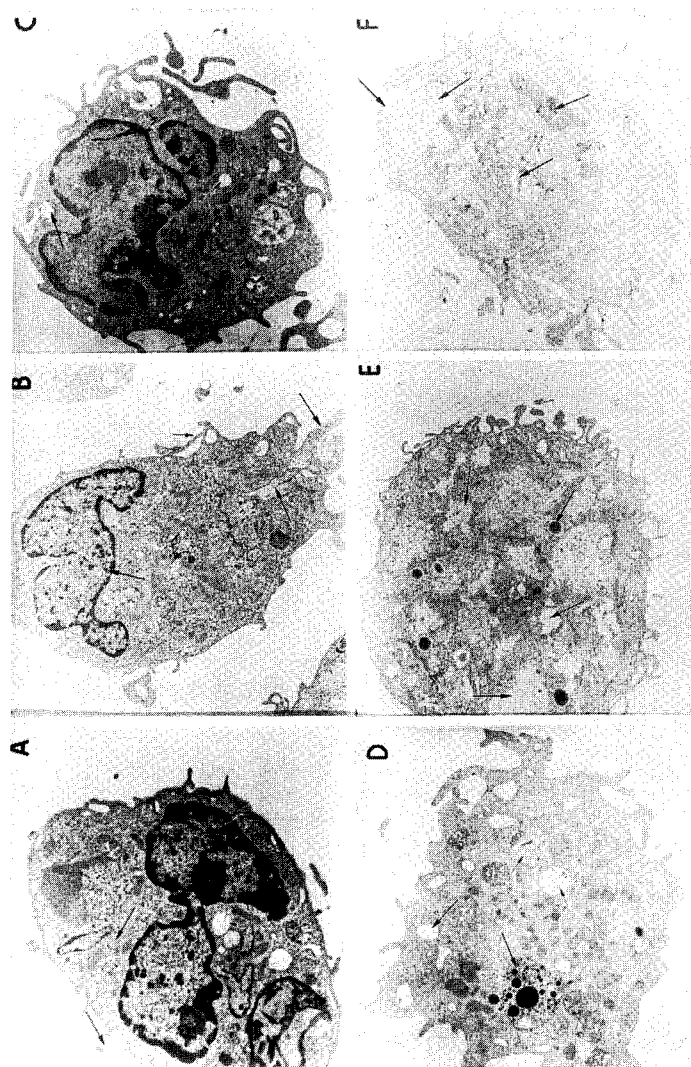
FIGURE 4. Electron microscopic studies of human peritoneal macrophages (MØ). **A:** Control MØ incubated without Hb. **B:** MØ incubated with UHb. **C:** MØ incubated with LMWHb. **D:** MØ incubated with HMWHb. **E:** MØ incubated with Hb contaminated with stromal lipids. **F:** MØ incubated with Hb contaminated with endotoxin. (Morphological features described in the text. Print magnification: x 16,500).

DISCUSSION

The role of cytokines as mediators of various biological responses including inflammation, immunological reactions and erythropoiesis is well documented in the scientific literature. It was reported that many systemic reactions may be mediated by these potent stimulatory products of various cells, especially the RES cell system [2]. To date there is a lack of information on stimulatory activities of Hb on cytokines release by the RES system. One scientific report has shown that pure HbAo may stimulate M to release IL-8 [14].

The results of the present study demonstrate that human M and MØ-L may release numerous cytokines following stimulation with Hb. The kinetics of cytokine expression was different for M and MØ-L colonies. The stimulation of cytokine release by Hb from M was shown to be a more long-lasting process, while the MØ-L response was much faster. Perhaps, peritoneal MØ-L associated with endometriosis are endowed with some degree of activation. The unmodified Hb may stimulate the release of TNF from M, and TNF, IL-1 and IL-6 from MØ-L. Our study shows that proper, effective chemical modification of the Hb surface by our developed cross-linking substance, which results in formation of low M.W. polymers (<400 kDa), may decrease formation of IL-1, and stabilize TNF level in MØ-L cultures. On the other hand, improper Hb modification with formation of high M.W. biopolymers (<1,020 kDa) produced greater cell activation and caused significant increases of TNF and IL-1 and decreased GM-CSF production.

The observed Hb action on cytokine expression can be explained only on a theoretical basis. As reported earlier from our laboratory, the possibility of protein-protein interaction between Hb and non-Hb proteins/polypeptides, could help explain the direct and active role of Hb in stimulating certain cytokines receptors [15]. The fact that Hb represents such activity creates the possibility that this molecule may mimic actions of other well known stimuli, and could react directly with the surface receptors. Polymerization of Hb with formation of high M.W. biopolymers may introduce more chances for such direct external stimulation (interaction). Moreover, intracellular stimulation by Hb is also possible, as the RES cell system appears at some point to be spared in terms of Hb accumulation [16]. Our unpublished studies showed that large Hb polymers (>400 kDa) may increase intracellular (murine MØ) Hb accumulation. Furthermore, Hb was found to generate free oxygen radicals [17], and these active oxygen species can directly activate the TNF receptor [18].

Relatively low stimuli introduced by Hb may be amplified by bacterial and lipid contaminants. It has been reported that the lipid A portion of LPS may induce expression of TNF and IL-1 genes through stimulation of receptors on the surface of M and MØ cells [19]. The presence of pools of TNF mRNA and IL-1 mRNA may speed up this reaction and release of cytokines may occure within minutes [20].

Our observation that Hb may be involved in activation of M/MØ-L system to express cytokines introduces new understandings of Hb molecular activities. The Hb-protein interactions and the possibility of activation of certain surface receptors could signify that the Hb molecule is able to be involved in indirect biological reactions, with many functional consequences at the systemic level.

We believe that the observed stimulation of M and MØ-L by LMWHb to release TNF can be considered as physiological. Because this Hb solution can inhibit the production of GM-

CSF by M/MØ-L cells, a slightly higher expression of TNF could activate *in vivo* the endothelial cells to produce a sufficient amount of GM-CSF required for red marrow cell erythropoietic functions [21, 22]. On the contrary, over activation of MØ-L by high M.W. Hb polymers, bacterial and lipid contaminants may stimulate large releases of TNF and IL-1, which could inhibit erythropoietic effects and introduce massive systemic reactions. Preliminary work from our laboratory suggests that purity, polymeric status and Hb concentration may affect erythropoietic capability.

Hb+PLs, HMWHb, and Hb+E were found to simulate platelet activating factor production by peritoneal MØ-L. This active phospholipid exerts diverse biological actions and plays a role both in normal physiological events and a variety of pathological responses [3]. Probably, increased pools of PLs in MØ-L culture during the incubation with Hb contaminated by lipid impurities increased the possibility of PAF production. It is also quite possible that endotoxin or large Hb polymers may increase PAF synthesis by activation of PLA_2.

A number of studies have suggested that the cell and organ dysfunction associated with multiple organ failure (MOF) can be mediated by cytokines and PAF released primarily from activated M and MØ-L. Biological and metabolic effects of cytokines are correlated generally with hemodynamic disturbances, induction of fever, increases in procoagulant activity on the endothelial cell surface, activation of PLA_2, stimulation of prostaglandin and leukotriene formation, production of more cytokines, activation of blood neutrophils, and free oxygen radical induction [2]. Elevated levels of PAF can introduce systemic hemodynamic changes, myocardial injury with arrythmias, and renal, gastrointestinal and lung injury [3]. These and similar events have been reported by many researchers after the administration of various Hb preparations [23].

It is clear that for better understanding of pathophysiological events following intravenous administration of Hb solutions, more scientific attention must focus on Hb's involvement in cytokines synthesis *in vivo* , because their expression *in vitro* has been clearly documented.

CONCLUSIONS

1. Hb can be classified as an active molecule, able to stimulate M/MØ-L to express cytokines.

2. The stimulation of cytokines release by Hb from M was shown to be a more long-lasting process, while MØ-L response was much faster. UHb possessed an ability to release a significant level of various cytokines, especially TNF. Incubation of M and MØ-L with LMWHb resulted in inhibition of IL-1 production and stabilization of the TNF level. Large Hb polymers, endotoxin and lipid contaminants amplified cytokines expression.

3. Hb with high M.W. polymers, and Hb plus endotoxin and phospholipids contaminants were found to stimulate the production of PAF by the MØ-L.

4. The changes in cytokine expression introduced by LMWHb with chemically modified surface can be classified as beneficial. Decreased levels of IL-1 may contribute to the elimination of inflammatory reactions, but slightly increased levels of TNF, probably via expression of GM-CSF from endothelial cells, could introduce more effective erythropoiesis.

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HEMOGLOBINURIA IN RATS:A SENSITIVE TEST OF RENAL FILTERING AND ABSORPTION OF PEG-HEMOGLOBIN, A RED BLOOD CELL SUBSTITUTE.

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ABSTRACT

Hemoglobinuria, defined as hemoglobin or hemoglobin subunits in the urine, is an easily monitored, sensitive indicator of renal handling of hemoglobin-based blood substitutes. Hemoglobin tetramer dissociation increases filtration by the kidneys. When the rate of filtration exceeds reabsorption, hemoglobinuria occurs. This study investigates the renal filtration and absorption of polyethylene glycol-modified bovine hemoglobin by monitoring for hemoglobinuria in several model systems.

OBJECTIVES

The glomeruli of the nephron are ultrafiltration units responsible for, among other things, the filtration of the products of protein catabolism. Proteins of up to 65,000 daltons are filtered out of the plasma. Hemoglobin, which is normally encapsulated in the red blood cell as a tetramer (four 16,000 subunits, 2 alpha and 2 beta), is protected from glomerular filtration. Free hemoglobin, with its tendency to dissociate from its tetramer to dimer configuration, is readily filtered and excreted into the urine. We have modified bovine hemoglobin with polyethylene glycol (PEG-Hb) to produce a molecule with a molecular weight of greater than 100,000 daltons.

Hemoglobinuria, defined as hemoglobin, or its subunits, in the urine, produces coloration of the urine ranging from pink to a deep red. This condition is easily

monitored and is an extremely sensitive indicator of renal processing of hemoglobin-based blood substitutes. Hemoglobin can be detected in the urine at levels less than 0.02mg/ml [Brown, 1988]. We have used this sensitive in vivo method as a screen for molecular instabilities in PEG-conjugated Hb.

By using hemoglobinuria as a development tool, we have produced a PEG-Hb that is not filtered through the rat kidney in an exchange transfusion model and has an extended circulating half-life.

METHODS

Adult male or female Sprague-Dawley rats (300-375gm) were anesthetized with a 3:1 mixture of Ketamine:Xylazine diluted 1:1 with dH$_2$O via i.p. injection at 0.2ml/100gm.

Exchange Transfusion Procedure

Rats, femoral veins cannulated, were bled with immediate infusion of equivalent volumes of PEG-Hb (1ml/minute until the appropriate replacement volume was achieved).

Volume exchange was equal to body weight x 0.06 (percent blood volume in the rat) x percent volume replacement, i.e., to achieve a 50% ET in a 300gm rat, the calculation would be 300gm x 0.06 x 0.5 = 9ml.

Bolus Infusion Procedure

PEG-Hb was infused as a bolus into the rat, via 25 gauge butterfly needle inserted into the tail vein, at a rate of 0.2ml/minute to a low dose of 12ml/kg or a high dose of 25ml/kg.

After procedures were performed, rats were placed in metabolism cages and urine was collected at 24h, 48h, and 72h.

Hemoglobin concentrations were determined using a Hemoximeter OSM-3 (Radiometer).

RESULTS

Purified bovine hemoglobin was conjugated to polyethylene glycol and the modified hemoglobin was processed to remove any unreacted hemoglobin or polymer (Figure 1). Before selecting a final formulation for our hemoglobin-based blood substitute, we screened a number of different preparations.

In the rat exchange transfusion model, only the PEG-Hb preparation with an average molecular weight of 110 kD produced hemoglobinuria, which was transient in nature (Table I). The higher molecular weight PEG-Hb preparations (average molecular weight $\geq$ 120 kD) did not produce detectable hemoglobinuria.

As expected, the hypervolemic model, in which the rat was infused with PEG-Hb up to an additional 35% of blood volume over a 20 minute period, produced both volume stress and protein overload resulting in hemoglobin leakage into the urine with preparations of the PEG-Hb (Table II). However, in several of the preparations (average molecular weights $\geq$ 120 kD) the hemoglobinuria detected was less than two percent of the administered dose (Figure 2).

PEG-Hb, with its high molecular weight and increased radius, demonstrated a circulating half-life (t 1/2) in bolus injected rats of 17-19 hours. The t 1/2 of two such preparations of PEG-Hb were measured (Figure 3). The t 1/2's of the PEG-Hb samples (average molecular weight 120 kD) were similar, even though bolus #1 did not show hemoglobinuria, and bolus #2 and bolus #3 showed less than two percent hemoglobinuria. This suggests that slight hemoglobinuria (< 2% of the total dose) does not affect the t 1/2 of the PEG-Hb product. The size of the PEG-Hb molecule appears to play a more important role in hemoglobinuria.

DISCUSSION

Measuring hemoglobinuria is a rapid and sensitive in vivo method to monitor hemoglobin-based blood substitute products for filterable break-down products.

```
                         RBC Collection
                           40 Liters
                Hb Harvest      │
         Day 1                  ▼              Yield 55%
                         Chromatography
                                │
         Day 2                  ▼              Yield 95%
                          Viral Removal
                                │
         Day 3                  ▼              Yield 90%
                           Pegylation
                                │
         Day 4                  ▼              Yield 100%
                          Formulation
                                │
         Day 5                  ▼              Yield 93%
                          Bulk Yield 33%
                            210 units
```
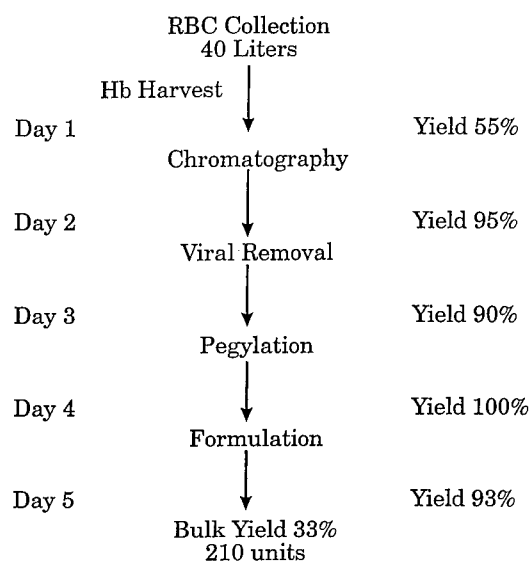
FIGURE 1.   PERCENT YIELDS FROM EACH PROCESSING STEP OVER
            A PERIOD OF 5 PROCESSING DAYS.

TABLE I.   Hemoglobinuria in Exchange Transfused Rats using PEG-Modified
            Bovine Hemoglobin.

Sample	Percent Exchange Transfusion	Hemoglobinuria
PEG-Hb Average MW=110 KD	50% ET	1/3
PEG-Hb Average MW=120 KD	30% ET	0/3
	50% ET	0/6
	70% ET	0/6
PEG-Hb Average MW=130 KD	50% ET	0/12

TABLE II.   Hemoglobinuria in Hypervolemic (Bolus) Infused Rats using PEG-Modified Bovine Hemoglobin.

Sample	Bolus	Hemoglobinuria
PEG-Hb Average MW = 110 KD	25 ml/Kg	2/3
PEG-Hb Average MW = 120 KD	12 ml/Kg 25 ml/Kg	2/6 6/11
PEG-Hb Average MW = 130 KD	25 ml/Kg	13/16
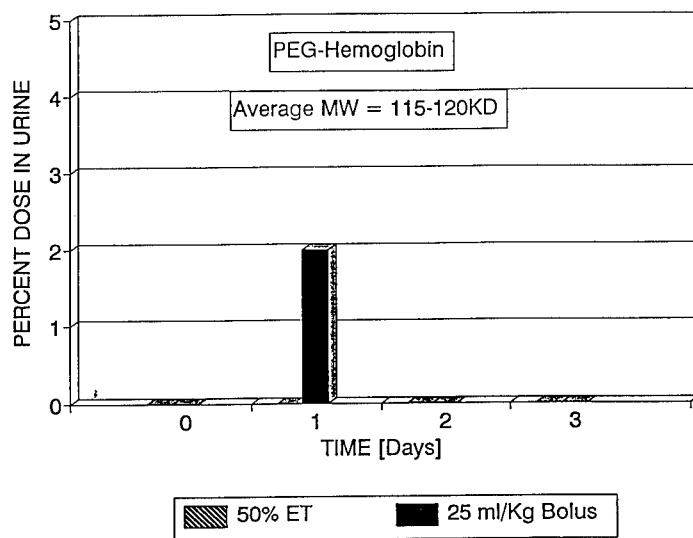
FIGURE 2.   TIME COURSE OF PEG-HB INDUCED HEMOGLOBINURIA IN A BOLUS MODEL.
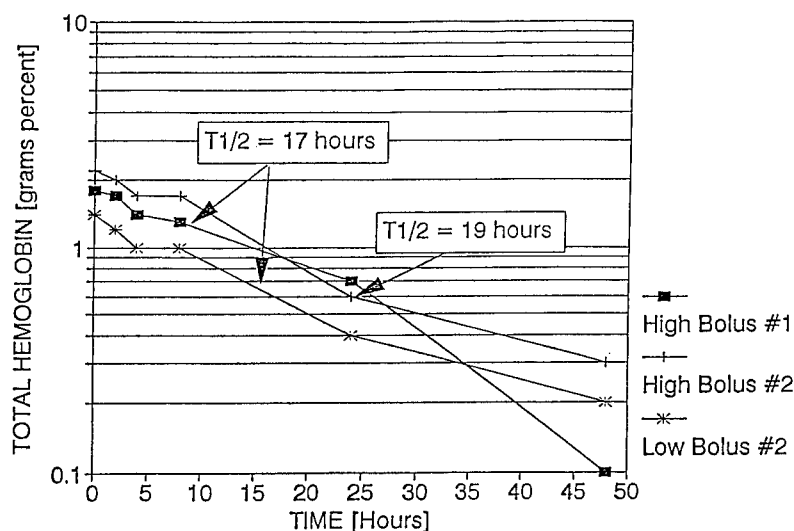
#1 25 ml/kg of PEG-Hb, average MW 120 kD, no hemoglobinuria.
#2 25 ml/kg of PEG-Hb, average MW 120 kD, hemoglobinuria.
#3 12 ml/kg of PEG-Hb, average MW 120 kD, slight hemoglobinuria.

FIGURE 3.   PEG-HB HALF-LIFE STUDY (BOLUS INJECTION)

Results can be obtained in less than 24 hours. We have shown that as little as 0.04% leakage can be detected in rat exchange transfusion or hypervolemic models. The formula for calculating percent leakage in a 50% exchange transfused rat is: 0.02mg/ml (minimum detectable levels of hemoglobin) x 10ml (average daily rat urinary output)/540mg (total infused PEG-Hb in the 50% model).

The size and structure of PEG-Hb prevents its filtration by the kidney and prolongs its circulating half-life. Most preparations of PEG-Hb demonstrate no evidence of hemoglobinuria in exchange transfused rats, and only transient hemoglobinuria is observed in hypervolemic infused rats. This indicates that the PEG-Hb preparations are very stable and have minimal breakdown products. The

higher the PEG-Hb average molecular weight, the lower the chances of hemoglobinuria in the ET model. It is only in the hypervolemic model that PEG-Hb size may play a role in the hemoglobin's stability.

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Human Hemoglobin Conjugated to Carboxylate Dextran as a Potential Red Blood Cell Substitute.
-II- Pharmacotoxicological Evaluation.

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ABSTRACT

A solution of human hemoglobin bound to benzene tetracarboxylate substituted dextran, whose physicochemical characteristics are defined in part I, was evaluated in vivo as a potential red blood cell substitute [1]. Further experiments show :
 - the confirmation of a lack of acute toxicity in mice and guinea pigs after injection of 12.5%, 25% and 50% of the blood mass and the absence of death in rabbits having undergone three successive 25% hemorrhagic shocks in three week intervals.
 - a plasma half-life of 9.5 ± 0.5 hours in 70-75% hemorrhagic shocks on guinea pigs and the absence of dex-BTC-Hb in thoracic and abdominal cavities. No tissue oedema was noticed. Total hemoglobinuria did not exceed 10 % of the injected hemoglobin quantity and only involved free hemoglobin.
 - a lack of death in 70-75 % hemorrhagic shocks and survival times ranging from 10 hours to 3 days in total exchange transfusions in guinea pig experiments.

INTRODUCTION

At present, in the event of a like threatening of red blood cells, blood transfusion is a must, although its use is not without risks. To extend the function of plasma substitutes, which allow the recovery of blood volume but do not carry oxygen, unmodified hemoglobins (Hb) must overcome two main limitations : a high affinity for oxygen and a weak vascular remanence [2]. Our team has developed a potential human hemoglobin oxygen carrier whose aim is to ensure both the oxyphoric function and the volemic restoration .

The prepared product is a hemoglobin modified by covalent binding with benzene tetracarboxylate dextran (dex-BTC-Hb) with a molecular weight distribution centered around 300 kDa [3]; whose solution meets all the in vitro requirements for infusion in animals.

This report will feature the latest results of the pharmacotoxicologic study of this solution : the observation of its absence of toxicity in mice and guinea pigs, the determination of its plasma half-life, the evaluation of its oxyphoric capacity and of its volemic restoration capability by means of hemorrhagic shocks and partial or total exchange transfusions in guinea pigs.

MATERIALS AND METHODS

1) Preparation, analysis and characteristics of the different solutions

The preparation of hemoglobin conjugated to benzene tetracarboxylate dextran was described in part I. It was adjusted to 70 g/l of Hb, then stored in Tyrode's solution at - 20 °C before use. Its main characteristics are listed in table I. Measured according to usual methods, they have been published previously [3]. The results obtained with this solution were compared to those of 70 g/l unmodified purified hemoglobin (Hb) (table I) and 50 g/l purified human albumin solutions.

Only in the case of safety tests, we also used control solutions constituted of compounds used in the conjugated product (T 10 dextran and dex-BTC) with or without hemoglobin. All the solutions were identically isotonic in Tyrode's, sterile and pyrogen-free.

2) Animal experiments

They were carried out on Swiss mice (IFFA-CREDO, France), Hartley VAF guinea pigs (Charles River, France) and Fauve de Bourgogne rabbits (Elevage Scientifique

TABLE I : Physicochemical characteristis of the solutions studied.

	SaO$_2$ %	MetHb %	P$_{50}$ torr	Hill's plot	ΔO$_2$* ml/g	Viscosity cSt	Oncotic P. torr
dex-BTC-Hb	±98	< 5	21	2.50	0.30	1.90	30
Hb	98	< 1	14	2.59	0.07	0.88	23
Blood	98	< 1	27	2.36	0.32	1.9-2.3	24-28

* Oxygen volume released by one g of Hb after a PO2 decrease of 100 to 40 torr.

des Dombes, France) according to the following protocols :

- Safety tests in the mouse (n=10) and the guinea pig (n=5) : 12.2%, 25% and 50% of the blood mass of the studied solution were injected intravenously in a surplus volume. The animals' behaviour after injection, the evolution of their body weight and their absorption of water were observed during the next three weeks. After sacrifice, we weighed organs (heart, spleen, liver, lungs and kidneys). We also measured the bile volume (guinea pig) and we looked out for leaks of hemoglobin in body cavities.

- Safety tests in the rabbit : we performed three successive hemorrhagic shocks of 25 % of blood volume [1] in three-week intervals. Each group of five animals received either dex-BTC-Hb, its own blood, Hb, or albumin.

- Evaluation of vascular remanence in the guinea pig by qualitative and quantitative analyses, at 2, 4, 8, 10, 12, 14, 22 and 26 hours, of circulating Hb, after partial isovolemic exchange transfusions (50% blood mass, n=8).

- Evaluation of volemic restoration and of body oxygenation in the guinea pig by

    . hemorrhagic shocks with the subtraction of 70-75% of blood volume [1]

    . 50% and 75% partial or total isovolemic exchange transfusions [4].

The parameters recorded during hemorrhagic shocks and exchange transfusions were:

- The mean arterial pressure (carotid), the electrocardiogram and respiratory rate (Physiograph DMP-4B Narco USA), only during blood exchanges.

- The plasma parameters : Hb, metHb, oxygen capacity (Co-oxymeter, IL 482), blood gas (ABL 2, Radiometer). Only those showing a change will be given in the results.

- The urinary hemoglobin loss, in each experiment.

- The stability of dex-BTC-Hb in blood and urine samples which was studied by HPLC analysis (Varian LC 5020, UV 100 detector, TSK G 3000 SW column (Tosohas), reading at 403 nm, phosphate buffer 50 mM, pH 7.2).
- The possible presence of hemoglobin in the abdominal and thoracic cavities or oedematous tissues at the autopsy of animals.

RESULTS

- Safety test in the mouse and the guinea pig. No death was observed. The animal's behaviour, its ponderal evolution and water absorption were similar to controls. The weight of vital organs was unchanged (figure 1) and the bile volume was comparable.

- Safety test in rabbit. dex-BTC-Hb or its own blood did not lead to death and did not induce any anaphylactoïd reaction; whereas the other solutions (unmodified Hb and albumin) led to the death of the animals in the course of the second or third shock.

- Evaluation of vascular remanence in the guinea pig. 50 % of dex-BTC-Hb was still present in the vessels after 9.5 ± 0.5 hours (1.5h for unmodified Hb). The chromatographic analyses revealed a good cohesion of the conjugate after several hours of circulation. At first an important decrease of free hemoglobin deriving from unconjugate Hb in the solution administered appeared. It was then followed by a weak regular presence of free Hb, which evidenced a gradual breaking of dextran/Hb binding.

- Evaluation of volemic restoration and body oxygenation. After hemorrhagic shock, all the animals receiving dex-BTC-Hb survived (n=7). Motor activity returned and normal feeding were rapidly observed. No survival was obtained with unmodified Hb (n=3), or albumin (n=6).

In partial isovolemic exchanges (50 and 70-75%), all animals survived. In total isovolemic exchanges, survival times of 10 min. (n=5) with albumin, of 13 h (n=5) with unmodified Hb and of 9 h to 3.5 days (n=10) with dex-BTC-Hb, were obtained.

- The recorded parameters were

. Mean arterial pressure: it decreased during hemorrhagic shock, but was immediately compensated for as soon as the beginning of the reinjection of dex-
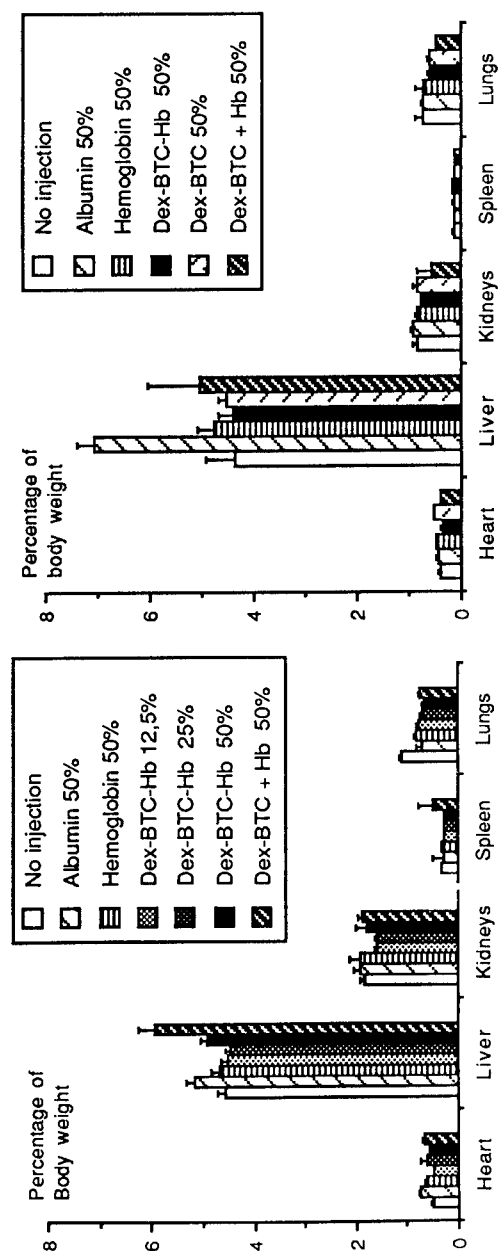
FIGURE 1 : Weight of main organs (in percentage of body weight) in guinea pigs (on right, n=5) and mice (on left, n=10), three weeks after intravenous injection of the solutions (mean±SEM).

BTC-Hb. With albumin, the pressure remained slightly inferior to its original value, whereas with all Hb solutions (conjugate or unmodified), it was slightly superior (+10 mmHg, i.e.20% of hypertension). The ECG and respiratory rate were only slightly modified with all solutions.

. Blood parameters: they were unmodified during and after shocks and exchanges, except PO2 which was increased by all Hb solutions (conjugate or unmodified) as long as a sufficient concentration of Hb stayed in the vessels.

. Percentage of urinary hemoglobin loss: it was similar in the hemorrhagic shock and the blood exchange. In hemorrhagic shocks, it reached 6.5 % (n=5) as opposed to nearby 15 % with unmodified Hb at the death of the animals, some 8 hours after the shock (n=3). For a 50 % exchange, it was 7.3 % (n = 3) and for this of 75 % , 11 % (n=3). In urines, Hb was especially present in a free or slightly conjugated form with less than 5 % of its molecular mass superior to 65 kDa.

. Possible presence of hemoglobin: no Hb was found during autopsy observations, in the abdominal and thoracic cavities; no oedema was present in tissues, especially in the intestine. So it seemed that the conjugate has reduced distribution through the vascular pores, compared to unmodified Hb.

DISCUSSION

The experiments in animals, carried out with this dextran-benzene-tetracarboxylate hemoglobin, co ifirmed the previous results [1]. The solution shows a lack of acute toxicity, even at high dosage. It allows an acceptable oxygen release into the tissues and remain in v ssels for a significant length of time with a plasma half-time close to 10 hours in guinea pigs; the conjugate extravasation of this product is moderate. Moreover it would seem that there is a limited gradual breaking of the dextran/Hb complex whose subproducts have been shown not toxic. Definitive survivals with hemorrhagic shocks and prolonged survival times with total exchange transfusions demonstrated the oxyphoric ability and the volemic restoration capacity of the solution.
These encouraging results lead us to compare important parameters in current literature like the plasma half-time which was between 5-8 hours in rats for DBBF [5], 10h in rats for o Raffinose polyhemoglobin [6], 12h in dogs for poly HbPLP [7] and 15h in rats for HbNFPLP [7], or the urinary hemoglobin loss which did

not exceed 6.5% in shocks of total perfused dex-BTC-Hb in shocks, whereas it was about 2.5% in rats for DBBF [5] and 1.4% in rats for poly NFPLP [7]. All the above values are relatively similar. For the other hemoglobins, results concerning hemorrhagic shocks and exchange transfusions have not been published recently and comparisons are not possible.

Dex-BTC-Hb solution appears to achieve the aims which were sought for insuring a volemic restoration and an oxygen transport. It can therefore be considered as a potential plasma and blood cell substitute.

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METHEMOGLOBIN FORMATION AFTER ADMINISTRATION OF
HEMOGLOBIN CONJUGATED TO CARBOXYLATE DEXTRAN
IN GUINEA PIGS.
ATTEMPTS TO PREVENT THE OXIDATION OF HEMOGLOBIN.

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ABSTRACT

In 1990, McGown demonstrated in vitro a limitation of extracellular methemoglobin (metHb) formation by releasing and recycling of ascorbic acid by red blood cells [1]. In order to investigate the autoxidation of free or modified hemoglobin in plasma and the possibility of reproducing McGown's phenomenon in vivo, we performed a 50% blood mass exchange in guinea-pigs with a 70±5 g/l dex-BTC-Hb solution (metHb<5%). Methemoglobin was determined according to Evelyn-Malloy's method. We observed a clear but limited oxidation of plasmatic hemoglobin (MetHb~30-40% at t=12hrs up to t=24hrs). A similar blood mass exchange was performed with the same hemoglobin solution which was previously totally oxidized into metHb. 40% of this methemoglobin was found to be reduced after 12hrs. These results demonstrated a marked reducing activity by residual blood as shown by others [2]. The addition of potentially protective compounds such as ascorbic acid (non enzymatic intraerythrocytar reduction pathway), methylene blue or riboflavin (enzymatic intraerythrocytar pathway), allowed a significant drop in the methemoglobin level. On the contrary, we didn't observe any reducing effect with reduced glutathione.

INTRODUCTION

Human hemoglobin extracted from erythrocytes has physicochemical characteristics unsuitable for unrefined therapeutic use. The chemical modifications of this molecule by conjugation to benzene tetracarboxylate-dextran (dex-BTC) restore its physiological characteristics in vitro : P50, Hill's number, oxidation state, molecular structure... all testify to the recovery of the oxygenation capacity. In an isotonic solution, this molecule is a potential temporary substitute for erythrocyte and plasma. In vitro, the physicochemical characteristics of this solution were controlled but its behaviour in vivo was unknown.

In this study, we only investigated the hemoglobin oxidation state which influence P50 and the hemoglobin oxygenation capacity. During the course of hemoglobin circulation through the vessels and its elimination, the oxidation state was influenced by the presence of oxidant and reducing systems and numerous other molecules in residual blood but also by the hemoglobin chemical modifications. We studied the benzene tetracarboxylate dextran-hemoglobin (dex-BTC-Hb) oxidation rate in vivo during 36h, the existence of systems which limit the circulating methemoglobin rate, as those found in erythrocyte, and we investigated what the involved antioxidant molecules were. For this purpose, we performed transfusional exchanges of 50% of blood volume with the dex-BTC-Hb solution in guinea pigs with a permanent catheter in the left femoral artery. This hemoglobin has a plasmatic half-life of about 10hrs [3]. Two types of dex-BTC-Hb solution were used : a solution with metHb <5% or one with metHb >90%. The tested antioxidants were ascorbic acid, riboflavin, methylene blue and reduced glutathione.

MATERIALS AND METHODS

Animals

This study was realised in Hartley V.A.F.® guinea pigs of 300-350 g (Charles River, St Aubain les Elbeuf, France). The femoral artery of these anaesthetized animals with Halothane and Carbogene, was catheterised by polyethylene catheter (Biotrol, France). This catheter filled with heparined physiologic saline was inserted under the skin and emerged at the neck. Animals were sutured and placed in a metabolic cage until they wake up.

Benzene tetracarboxylate dextran-hemoglobin solution (dex-BTC-Hb)

The characteristics of this solution are as follows : [Hb] = 70 ± 5g/l, P50 = 20- 22 torr, Hill number = 1.8-2.1, oncotic pressure = 32-35 torr, metHb <5%, pH 7.40

± 0.02. This solution was purified, sterile, pyrogene free and isotonised in Tyrode [4]. An oxidized solution, with a methemoglobin content >90%, was obtained by incubation at 37°C for 3 to 4 days.

Transfusional exchange of 50% of blood volume

Blood of the vigil animal was exchanged with dex-BTC-Hb solution by fractions of 2 - 2.5 ml to prevent significant haemodynamic disturbances. The removal of blood and the Hb solution injections were carried out slowly with sterile syringes. Times for Hb injection and subsequent removal of "blood" were at 3 or 4 minutes intervals. The exchange ended when the desired blood volume was extracted. The Hb extracted during exchange was taken into account to manage the exchange. The hematocrit was measured before and after the exchange in order to check the exchange extent. This protocol was performed with the two types of Hb solutions without subsequent treatment, and with non-oxidized Hb solution with various protective treatments.

Blood sampling and analyses

The first sample was taken as early as the end of the transfusional exchange (t=0). One part was homogenized and analysed directly, the other part was centrifuged at 2500g, 10 min at 4°C.

The parameters measured :

- on whole blood were : the total circulating hemoglobin concentration, percentages of HbO_2, HbC0, MetHb and the vol%O_2 on Co-oxymeter IL 282 (Instrumentation Laboratory, USA), and the blood gas on ABL2 (Radiometer, Copenhague) in order to verify the absence of strong blood disturbances after transfusional exchange.

- on the supernatant (plasma and injected dex-BTC-Hb) were : the plasmatic dex-BTC-Hb concentration with Drabkin's method and the dex-BTC-methemoglobin percentage with Evelyn and Malloy's method.

- on the culot (residual red blood cells after transfusional exchange) were : percentages of HbO_2, HbCO and MetHb on the Co-Oxymeter IL282 in order to verify oxyphoric properties of this cells.

Further samples taken during the 36hrs period (every 2hrs during 12hrs and then every 6hrs up to 36hrs) were centrifuged and analysed as mentioned above .

Antioxidants treatments

Ascorbic acid (2mmol per liter of blood, Laroscorbine 500mg®, Roche, France), riboflavin (0.3mmol per liter of blood, Béflavine® Roche, France), reduced glutathione (2.5mmol per liter of blood, Sigma, USA) and methylene blue (2mg/kg

b.w., prepared solution to 1%, RAL Prolabo, France) were injected into the femoral catheter at the end of exchange (t=0) or at t=4 and t=8hrs after exchange. Blood samplings and analyses were performed as mentioned above .

RESULTS

No chemical blood disturbance was observed during transfusional exchanges.

In vivo dex-BTC-Hb oxidation in plasma (figure $A_{1,2,3}$)

In the blood stream, 30 to 40% of dex-BTC-Hb was oxidized during the first 12 hours following the tranfusional exchange and this rate remained constant thereafter. The limited oxidized Hb rate observed after 12hrs in vivo could be the consequence of several phenomena : a) an equilibrium between the oxidation and the extravascular leak of Hb, b) the existence either of reducing systems which progressively limit the methemoglobin level, or c) the presence of protective systems which prevent dex-BTC-Hb oxidation, or d) the joint action of all these phenomena.

To uncover the existence of these systems, tranfusional exchanges were carried out with oxidized dex-BTC-Hb solution.

In vivo dex-BTC-methemoglobin reduction in plasma (figure B)

The methemoglobin injected into the blood stream underwent a rapid reduction of about 40% in 12hrs. This phenomenon evidenced the presence of plasmatic reducing systems.

The presence of large quantities of methemoglobin in the vascular system had a limited influence on red blood cells. The intraerythrocytar methemoglobin rate was of 3 to 4% during the first 4 hrs that followed the tranfusional exchange. Then it returned to its physiological values (<1,5%).

In vivo dex-BTC-Hb oxidation in plasma
- in the presence of ascorbic acid (figure A_1)

The injection of ascorbic acid in the blood stream as early as the end of the exchange did not prevent the fast oxidation of hemoglobin during the first 4 hours but afterwards it permitted a clear limitation of this up to 24hrs after the exchange (metHb <25%). The double injection at t=4 and t=8hrs did not improve this reduction. In fact it seemed to have the opposite effect.

- in the presence of flavin (figure A_2)

The effect of riboflavin depended on the timing of the injection. At t=0, this molecule had no effect on the circulating methemoglobin rate. On the contrary, its administration at t=4 and t=8hrs allowed a clear but transient reduction of the methemoglobin rate.

- in the presence of methylene blue (figure A_3)

This product did not interfere in the methemoglobin dosage with Evelyn and Malloy's method [5]. It strongly restricted the circulating plasmatic methemoglobin rate (metHb <15% at t=8hrs). A double injection at t=4 and t=8hrs did not improve the effect obtained with only one dose at t=0.

- in the presence of reduced glutathione

We observed no effect on the methemoglobin levels.

DISCUSSION

The hemoglobin substitute tested was subject to a strong in vivo oxidation in guinea pigs. In the presence of 50% residual red blood cells, this oxidation resulted in a 30 to 40% methemoglobin level after 12hrs of circulation in vivo. These results confirm those obtained in rats by den Boer and al [2]. A levelling off of the hemoglobin oxidation rate and a reduction of about 40% of injected methemoglobin enables us to conclude that reducing systems of circulating plasmatic methemoglobin do exist. Injected ascorbic acid had a good reducing capacity and remained efficient for about 24 hrs. As McGown demonstrated it in vitro, this efficiency can be explained by the recycling of ascorbic acid which has been oxidized during direct methemoglobin reduction, by red blood cells. We noted that the injection of high doses may have an opposite effect. Riboflavine and methylene blue were also efficient to reduce this plasmatic methemoglobin level. This reduction may be ascribed to an enzymatic pathway involving the NADPH dependent reductase present in liver and identical to the one in erythrocytes [6, 7]. Between the enzyme and the methemoglobin, this pathway needs the presence of an intermediate electron acceptor which can be a dye such as methylene blue or a flavin such as riboflavin, both absent or in very low quantity in the blood stream. Then methylene blue strongly limited the plasmatic methemoglobin rate as early as the end of transfusional exchange and for about 8hrs.
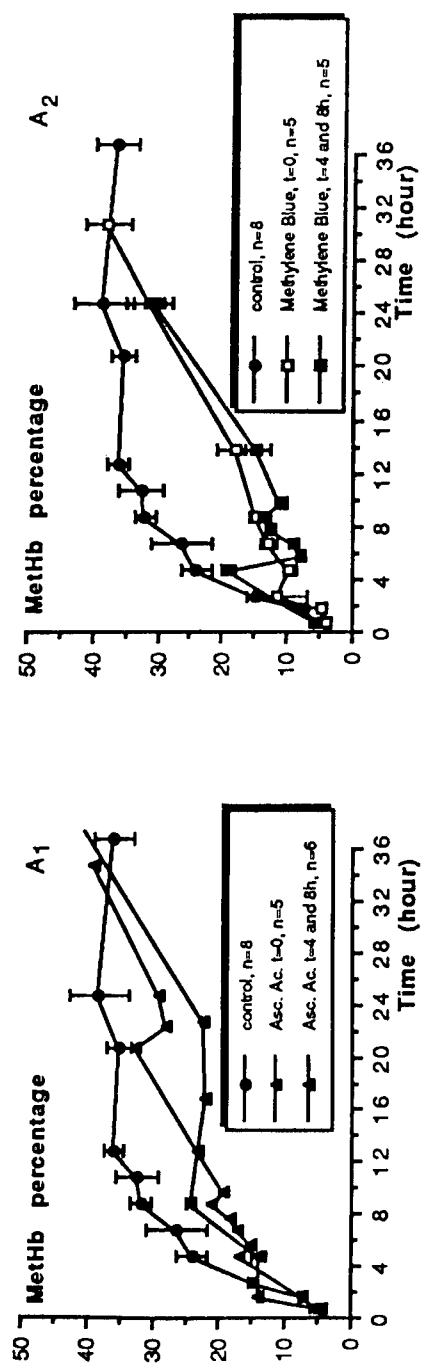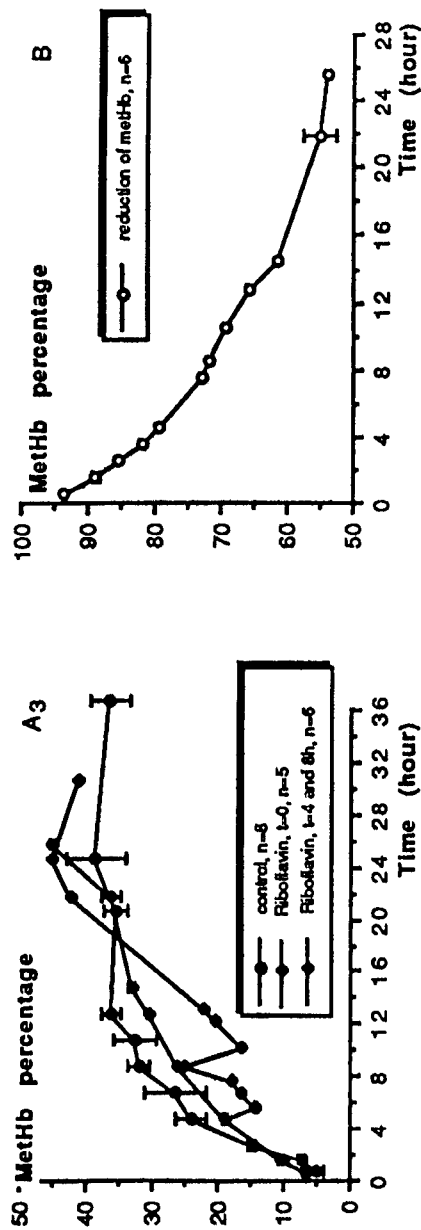
FIGURE A : Evolution of dex-BTC-Hb oxidation versus time in vivo after transfusional exchange in guinea pigs and in the presence of 1) ascorbic acid (2mmol per liter or blood) ; 2) methylene blue (2mg/100 g b.w.) and 3) riboflavine (0.3 mmol per liter of blood). FIGURE B : Evolution of methemoglobin-dex-BTC reduction versus time in vivo after transfusional exchange in guinea-pigs. Two antioxidant administration schemes : one administration at t=0 or two administrations at t=4hrs and t=8hrs.

Considering the reducing potential in the organism, hemoglobin based blood substitute injections should be accompanied by ascorbic acid for a non enzymatic reduction and especially by methylene blue for increasing enzymatic reduction which could take place in the liver as well as in red blood cells..

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ACELLULAR AND CELLULAR HEMOGLOBIN SOLUTIONS AS VASOCONSTRICTIVE FACTOR

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ABSTRACT

The inhibitory effects of acellular and cellular hemoglobin (Hb) solutions on endothelium-dependent vasorelaxation were investigated in rabbit thoracic aortic strips. As acellular Hb solutions, 2,3-diphosphoglycerate (DPG)-depleted Hb and pyridoxylated Hb were examined. Cellular Hb solutions included washed human fresh red cells and liposome Hb encapsulated with pyridoxal-5'-phosphate (PLP). The tissues were precontracted with phenylephrine (PE), after which acetylcholine (ACh) was added to elicit a steady-state relaxation. Acellular Hb solutions cumulatively reversed ACh-induced relaxation, and these inhibitory effects reached a plateau at 10 µg/ml. Increasing oxygen affinity by pyridoxylation had little effect on this. In contrast, both red cells and liposome Hb solution showed moderate inhibitory effects, and they reached a plateau at 1 mg/ml. These findings indicate that acellular Hb solutions are more potent inhibitors than cellular Hb solutions by a factor of about 100, and that the encapsulation of Hb is a preferable method to mimic the red cell.

INTRODUCTION

Vasoconstriction is one of the unexpected reactions induced by stroma-free hemoglobin (SFH) and chemically modified red cell substitutes [1]. Although the precise mechanisms involved in vasoconstriction remain to be elucidated, Hb itself has been considered to be a candidate vasoconstrictor, since it is a well-known

559

potent inhibitor of endothelium-derived relaxing factor (EDRF) [2], which plays an essential role in regulating vascular tone and other functions. However, the red cell may also act as an inhibitor for EDRF [3]. In the present study, we have therefore investigated the contractile activity of several Hb preparations including acellular and cellular Hb solutions in comparison with intact human fresh red cells.

MATERIALS AND METHODS

Preparation of Hb Solutions- Venous blood was obtained from healthy volunteers. Highly purified SFH was prepared from fresh red cells by the method of BMM filtration as described [4] and freed of DPG by Sephadex G-25 filtration [5]. This DPG-depleted Hb was then pyridoxylated in the deoxy conformation [6]. Unbound PLP was removed with the same column. For the preparation of liposome-encapsulated Hb, SFH was previously dialyzed against 25 mM HEPES, pH 7.4, and concentrated until the Hb concentration was 26 g/dl or more. After the addition of PLP and NADH (Hb:PLP:NADH=1:3:0.1 in molar ratio), the osmolarity was adjusted at 310 mOsm by adding solid NaCl. Liposome encapsulation was performed by an extrusion method with a phospholipid mixture containing DPPC/cholesterol/DMPG-Na$^+$ (5:3:2 in molar ratio). Uncapsulated Hb was removed by chromatography with Sepharose CL-4B. Red cells were washed with phosphate-buffered saline and used immediately.

In Vitro Experiments Using Rabbit Aortic Strips- New Zealand albino rabbits of either sex, 2.0-3.0 kg, were used to prepare thoracic aortic strips 3 mm in width. The tissues were mounted in an organ bath under 1 g of tension in HEPES solution (37°C) of the following composition (mM): HEPES 5.0, NaCl 143, KCl 5.8, CaCl$_2$ 2.5, MgCl$_2$ 1.2, glucose 11, pH 7.4, aerated with air. The tissues were precontracted with PE (1 mM), and ACh (1 mM) was added to elicit a steady-state relaxation. Concentration-response curves to Hb were then constructed by adding cumulative concentrations of Hb solutions (from 10 ng/ml to 1 mg/ml). The response to each concentration was expressed as a percentage of the maximal relaxation induced by ACh. In another experiment, the contractile activity of DPG-depleted Hb at 1 μg/ml was examined in the presence of superoxide dismutase (SOD, 100 U/ml) to eliminate superoxides which might be formed in the Hb solution.

RESULTS

Table I shows the characteristics of Hb solutions used in the present study. The diameter of liposome Hb was 499 ± 317 nm (mean ± SD), and the molar ratio of Hb to lipid was calculated to be 0.26, suggesting multilamelar liposomes.

TABLE I. Characteristics of hemoglobin solutions

Hb solution	Hb (g/dl)[a]	MetHb (%)	P_{50} (mmHg)	Hill coefficient
DPG-depleted Hb	7.2	0.4	9.5	2.3
Pyridoxylated Hb	11.4	1.8	20.3	2.4
Liposome-Hb	1.2	—[b]	23.3	2.9
Human red cells	20-22	1-1.5	29.5	2.8

[a] Hb concentration of the stock solutions.
[b] No peak or shoulder identical to methemoglobin (MetHb) found by spectrophotometric examination.

All Hb solutions examined in the present study produced a concentration-dependent inhibition of ACh-induced relaxation. Representative tracings of a typical experiment of each Hb solution are shown in Fig. 1. Liposomes containing HEPES solution instead of Hb showed no effect on ACh-induced relaxation. Acellular Hb solutions such as DPG-depleted Hb and pyridoxylated Hb strongly inhibited the responses evoked by ACh (Fig. 2). These inhibitory effects reached a plateau at 10 μg/ml. The pyridoxylation of DPG-depleted Hb had little effect on its contractile activity. On the other hand, cellular Hb solutions such as liposome Hb and human red cells showed moderate inhibitory effects, and they reached a plateau at 1 mg/ml (Fig. 2).

Free radicals could be formed in Hb solutions [7], and superoxides can degrade EDRF. Thus, similar experiments were performed in the presence of SOD. DPG-depleted Hb at 1 μg/ml inhibited ACh-induced relaxation by 70.2±3.8 % (n=5, mean±SE) in the absence of SOD, while it was inhibited by 74.1±5.8 % (n=6) in the presence of SOD, indicating that superoxides are not substantially involved in the Hb-induced inhibition of ACh-induced relaxation in this model.

DISCUSSION

The major findings emerging from this study can be summarized as follows: (a) red cells can act as a vasoconstrictor in vitro; (b) the contractile activity of liposome Hb is similar to that of fresh human red cells; in contrast, (c) acellular Hb solutions inhibit ACh-induced relaxation more potently than cellular Hb solutions by a factor of about 100; and (d) increasing oxygen affinity has little effect on the contractile activity of Hb.

The major differences between red cells and acellular Hb solutions are cellular partitioning of Hb molecules and oxygen affinity. Since pyridoxylated Hb showed
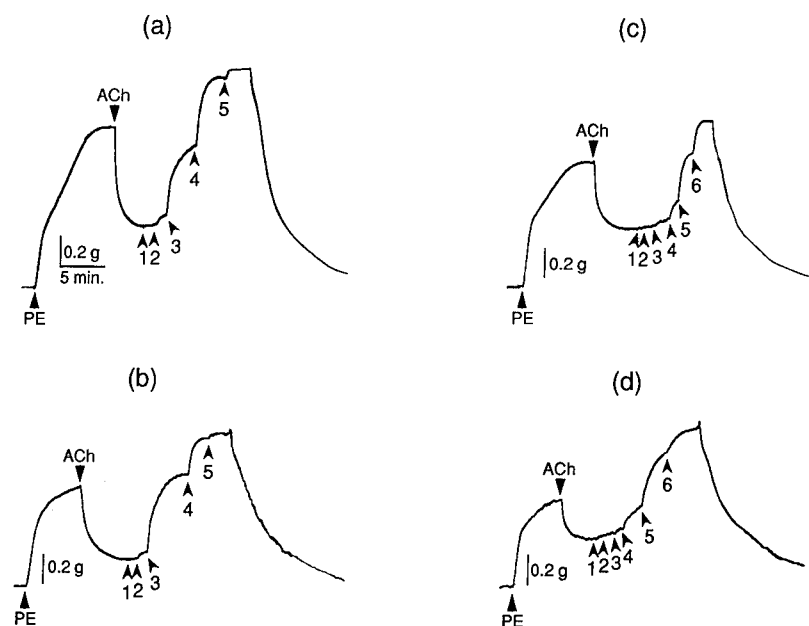
FIGURE 1   Typical experiments showing the effects of cumulatively added
hemoglobin solutions from 10 ng/ml to 1 mg/ml. (a) DPG-depleted
Hb, (b) pyridoxylated Hb, (c) liposome Hb, and (d) red cells. PE:
phenylephrine 1 μM; ACh: acetylcholine 1 μM. Hb concentrations at
10^n ng/ml.

the same inhibitory activity as DPG-depleted Hb, the difference in oxygen affinity
of about 10 mmHg is not significant for EDRF inactivation by Hb molecules.
Indeed, nitric oxide has about 1500 times greater affinity for Hb than has carbon
monoxide [8]. Therefore, cellular partitioning is thought to be the major factor
influencing the contractile activity of Hbs. The finding that liposome Hb solution
had the same activity as red cells reinforced this explanation. Acellular Hb
molecules can disperse freely, whereas Hbs in cellular vesicles are packed as a
dense solution. Although further studies on the contributions of the membrane
thickness and the shape and size of vesicles to EDRF inactivation by cellular Hb are
necessary, it is apparent that cell-free Hbs can bind nitric oxide more effectively
than packed Hbs.

Hb solutions may contain vasoconstrictors other than Hb [9,10]. Although the
possibility that cellular partitioning of Hbs can keep these factors in isolation and
that acellular Hbs thereby produce stronger inhibitions of ACh-induced relaxation
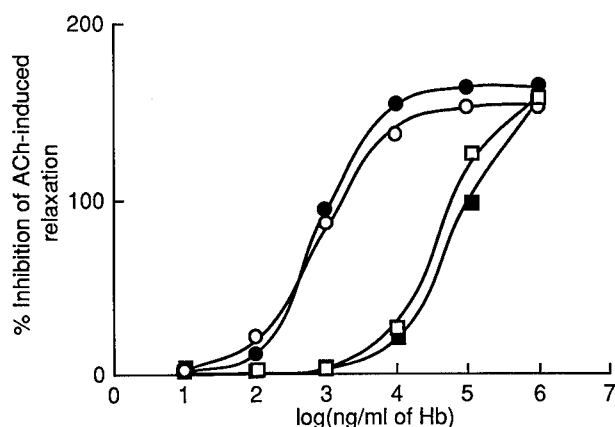
FIGURE 2   Inhibitions of ACh-induced relaxation by acellular and cellular Hb
            solutions. DPG-depleted Hb (filled circles), pyridoxylated Hb (open
            circles), liposome Hb (filled squares), and red cells (open squares).

cannot be excluded, the minimum Hb concentration at which acellular Hbs showed
contractile activity was in the range of 100 ng/ml. Acellular Hb solutions at these
concentrations had no direct contractile effect on the unprecontracted tissues (data
not shown). Superoxides can also induce constriction by degrading EDRF, and
such free radicals could be formed in Hb solutions [7]. However, elimination of
superoxides by SOD had no significant effect on constriction induced by DPG-
depleted Hb. These findings suggest that the effects of other vasoconstrictors are,
even if they are present, negligible, at least in this model.

The present study demonstrated that human intact red cells inhibited ACh-
induced relaxation in vitro, indicating that red cells also inactivate EDRF by binding
chemically. Therefore, the red cell can act as an EDRF inactivator in vivo. It is
possible that such a moderate inhibitory effect of red cells on EDRF is essential to
maintain the homeostasis in vascular tone and other functions associated with
EDRF. In this context, red cells inhibit EDRF, which is released into the lumen of
blood vessels, thus inhibiting its downstream effects. On the other hand, the effect
on EDRF released abluminally is small [3]. The observed potent inhibitory activity
of acellular Hb solutions leads to the proposal that they will inactivate EDRF
released into the lumen more effectively than red cells in vivo. This may account for
the vasoconstriction induced by SFH and acellular artificial oxygen carriers.
Alternatively, acellular Hb molecules enter into the interstitium and subsequently

into tissue cells, and inhibit EDRF released abluminally [1]. In any case, we found that encapsulated Hb had a contractile activity similar to that of red cells. Furthermore, encapsulated Hbs stay in the lumen of the vessel. Partitioning of Hb solutions with an artificial membrane will also prevent other possible vasoconstrictors and biologically active agents, if they exist in the Hb solutions, from interacting with endothelium and blood cells. Liposome encapsulation seems to be a preferable method to mimic the red cell.

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VASOCONSTRICTOR EFFECTS IN ISOLATED RABBIT HEART PERFUSED WITH BIS(3,5-DIBROMOSALICYL)FUMARATE CROSS-LINKED HEMOGLOBIN (ααHb)

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ABSTRACT

To study the mechanism by which cell-free hemoglobin preparations may alter coronary vascular reactivity, we investigated the effect of human hemoglobin cross-linked between alpha chains with bis(3,5-dibromosalicyl)fumarate (ααHb) on the vasomotor response to acetylcholine (ACh) in isolated perfused rabbit hearts. Dose-response curves were generated by monitoring the increase in coronary pressure during serial addition of 0.2-10 μM ACh before, during and after 20 min infusion of three test solutions: a) 0.1 g/dl ααHb (62 μM heme); b) 0.1 g/dl ααHb plus 60 μM deferoxamine (DFO); c) 50 μM N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of nitric oxide (NO) synthase. We found that the sensitivity to ACh-induced vasoconstriction was significantly potentiated in the presence of ααHb and L-NAME. In addition, this response was only partially reversed after removal of ααHb, except when DFO was simultaneously infused with the ααHb solution. These findings are consistent with the idea that both NO binding to hemoglobin and iron-mediated oxygen free radical generation contribute to an altered coronary vasomotor responsiveness induced by cell-free hemoglobin.

INTRODUCTION

Previous investigations have demonstrated that cell-free hemoglobin can be vasoactive *in vitro* [1,2] and *in vivo* [3]. Although coronary vasoconstriction has been reported when different human modified hemoglobins are infused at low concentration in isolated perfused hearts [4,5], the manner in which these preparations might alter coronary vasomotor responsiveness to endogenous vasoactive agents is unknown.

It has been suggested [6] that hemoglobin inhibits the relaxation of isolated aortic rings by interacting with the endothelium-derived relaxing factor (EDRF), a potent vasodilating agent that is generally thought to be nitric oxide (NO) [7]. It has also been suggested that hemoglobin-mediated toxicity might results from the generation of cytotoxic oxygen species accompanying autoxidation [8]. The presence of iron in the heme pocket and possible contamination with adventitious iron from non-hemoglobin sources create the possibility that preparations of hemoglobin might act as Fenton reagent [9] and induce free radical-mediated endothelial cell damage with subsequent changes in vascular smooth muscle tone [10].

In this study we tested the vasomotor response to acetylcholine (ACh) in isolated rabbit heart perfused with buffer containing human hemoglobin cross-linked between Lys-99α residues with bis(3,5-dibromosalicyl)fumarate ($\alpha\alpha$Hb). We examined the effect of hemoglobin on the vasomotor response to ACh in the presence and absence of the iron chelator deferoxamine mesylate (DFO). Additional experiments were run to determine if changes in ACh responsiveness induced by $\alpha\alpha$Hb could be mimicked by blocking endothelium-derived NO synthesis.

MATERIALS AND METHODS

Isolated perfused heart. Male New Zealand White rabbits (2.7-3.6 kg) were anesthetized, and their hearts were removed and perfused according to Langendorff as previously described [11]. The perfusion buffer contained (in mM): 117.4 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4 \cdot H_2O$, 1.2 KH_2PO_4, 0.03 Na_2EDTA, 24.6 $NaHCO_3$, 16.7 glucose (pH = 7.4). Indomethacin (10 μM) was added to the perfusate to prevent the cycloxygenase activity which is a source of both vasoconstrictors and vasodilators. The perfusate was equilibrated with

95% O_2/5% CO_2, and the temperature was maintained at 37° C. Hearts were electrically paced at 180 beats /min (4 V, 10 ms duration) and were perfused at constant flow rate (55.3±1.9 ml/min, n=14) in order to obtain an initial coronary pressure (CP) of 65-70 mmHg, which was monitored with a pressure transducer (Gould model P23 XL, Cleveland, Ohio) connected to the aortic cannula.

A latex balloon was inserted into the left ventricle through the left atrial appendage and connected to a second pressure transducer. The volume of the balloon was adjusted to achieve an end-diastolic pressure of 5-10 mmHg. CP and left ventricular function were continuously recorded on a strip chart recorder (2400S; Gould, Cleveland, OH).

Chemicals. Acetylcholine hydrochloride (ACh), indomethacin, deferoxamine mesylate (DFO) and NG-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. (St. Louis, MO). Human hemoglobin cross-linked between alpha chains was obtained by reacting deoxygenated stroma-free hemolysate with bis(3,5-dibromosalicyl)fumarate as previously described [12].

Experimental protocol. Hearts were allowed to stabilize at constant flow with oxygenated buffer for 20 min before treatments were begun. The vasomotor response of resistance vessels in the coronary vasculature was obtained by monitoring the changes in CP during cumulative additions of ACh (between 0.2 and 10 μM) into the buffer by means of a syringe pump connected to the perfusion line. Concentration-dependent vasoconstriction to ACh was assessed before, during and after 20 min infusion of: a) 0.1 g/dl (final concentration) $\alpha\alpha$Hb; b) 0.1 g/dl $\alpha\alpha$Hb plus 60 μM DFO; c) 50 μM L-NAME. At the end of each dose-response titration with ACh the CP returned to the pre-infusion levels within 1 minute. A recovery of 10 min duration was allowed to the hearts before the next treatment was started. The testing solutions were prefiltered through a 0.2 μm filter (Nalgene, Rochester, NY) and infused with an additional pump connected to the perfusion line.

RESULTS

Infusion of isolated rabbit hearts for 20 min with $\alpha\alpha$Hb (0.1 g/dl final concentration) resulted in an enhancement of the CP induced by cumulative
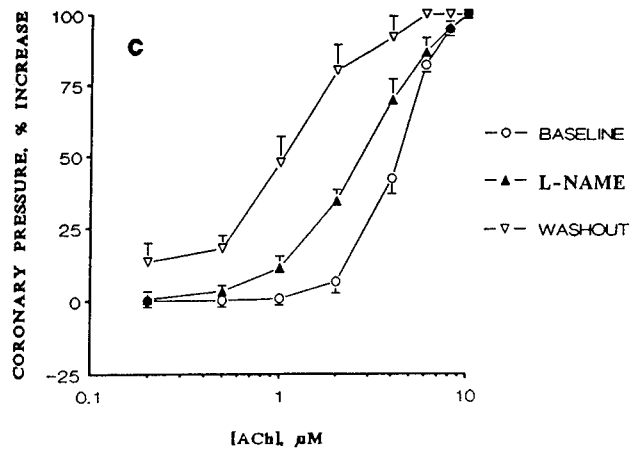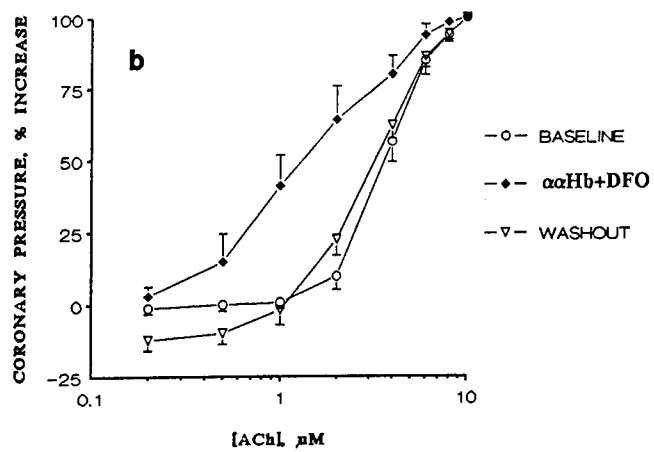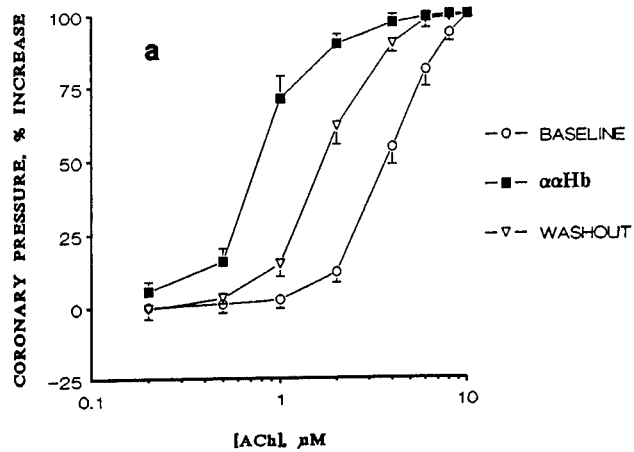
addition of 0.2-10 μM ACh (3.2 fold parallel shift to the left, n=5, p<0.05). This increase in ACh sensitivity was only partially reversible (2.1 fold shift from baseline) after removal of $\alpha\alpha$Hb by 20 min washout with the perfusion buffer (Fig. 1a). Addition of 60 μM DFO during infusion of hemoglobin significantly reduced the hemoglobin-mediated increase in ACh sensitivity (2.2 fold parallel shift to the left, n=5, p<0.05) and rendered it completely reversible after washout of $\alpha\alpha$Hb (Fig. 1b). Infusion of the NO synthase inhibitor L-NAME caused an initial increase in ACh sensitivity which was less than seen at any time during infusion of $\alpha\alpha$Hb (1.2 fold left shift in the dose-response curve, n=4, p<0.05), but which increased after washout to levels comparable to those observed with $\alpha\alpha$Hb plus DFO (2.7 fold shift from baseline, Fig. 1c).

The potentiation of ACh-induced coronary vasoconstriction following the infusion of $\alpha\alpha$Hb and L-NAME was associated with a greater depression of myocardial function at the end of the experimental protocols (Table 1). After 20 min washout of $\alpha\alpha$Hb, EDP and CP were significantly increased by 12.6±2.2 mmHg (Mean±SE) and 33.4±2.4 mmHg, respectively, whereas LVDP was decreased by 24.7±5.2% of the baseline value. Similarly, the infusion of L-NAME was associated with an increase by 16.5±3.7 mmHg in EDP and 41.5±4.5 mmHg in CP, whereas LVDP was significantly decreased by 23.3±5.0%. Simultaneous infusion of DFO with $\alpha\alpha$Hb induced a smaller increase in EDP (5.8±1.8 mmHg) and CP (15.0±2.2 mmHg) which were associated with smaller depression in LVDP (18.8±3.1%).

DISCUSSION

The results of this study are consistent with the idea that changes in steady-state distribution of endothelium-derived NO and iron-mediated generation of cytotoxic oxygen free radicals both contribute to altered

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FIGURE 1.   Dose-response curves to acetylcholine (ACh) before, during and after infusion of $\alpha\alpha$Hb (a), $\alpha\alpha$Hb plus DFO (b) and L-NAME (c). The increases in coronary pressure are expressed as a percentage of the initial value. The data are shown as mean of 4-5 observations with SEM indicated by vertical bars.

TABLE I. Hemodynamic parameters in isolated hearts perfused at constant flow before and after infusion of ααHb, L-NAME and ααHb+DFO.

GROUP	BASELINE	AFTER REMOVAL	DIFFERENCE
Coronary pressure (mmHg)			
ααHb(n=5)	64.6±2.8	98.0±2.9	33.4±2.4*§
L-NAME(n=4)	64.8±1.6	106.3±4.3	41.5±4.5*§
ααHb+DFO(n=5)	65.2±1.5	80.2±3.8	15.0±2.2*
End-diastolic pressure (mmHg)			
ααHb	7.8±0.9	20.4±2.2	12.6±2.2*§
L-NAME	9.8±0.6	26.3±3.7	16.5±3.7*§
ααHb+DFO	7.8±0.7	13.6±1.4	5.8±1.8*
Left ventricular developed pressure (% baseline)			
ααHb	100	75.3±5.2	24.7±5.2*
L-NAME	100	76.7±7.4	23.3±5.0*
ααHb+DFO	100	81.2±3.1	18.8±3.1*

Values are means ± SE. Constant coronary flow rates were maintained by means of a peristaltic roller pump. Fourteen hearts were studied and assigned to one of three different groups depending on the solution infused. The solutions were infused for 20 min with a syringe pump at constant flow rate to obtain the desired final concentration in the buffer perfusing the heart. Myocardial functions were recorded during perfusion at constant flow with Krebs Henseleit buffer (baseline) and 20 min after removal of the testing solutions with buffer returning to the baseline conditions. Groups: ααHb, 0.1 g/dl alpha-alpha cross-linked hemoglobin; L-NAME, 50 μM N^G-nitro-L-arginine methyl ester; ααHb+DFO, 0.1 g/dl ααHb plus 60 μM deferoxamine mesylate; *P<0.05 vs baseline; § p<0.05 vs ααHb+DFO group.

coronary vasomotor responsiveness induced by hemoglobin. The concentration-dependent vasoconstriction elicited by ACh in isolated rabbit hearts is intensified by the presence of $\alpha\alpha$Hb, and this response is significantly attenuated in the presence of an iron-chelating compound (DFO). The concept that NO participates in the normal steady-state responsiveness of this preparation to vasoactive agents and that hemoglobin-mediated interference with normal NO distribution alters this response is further bolstered by the increased sensitivity to ACh observed when endothelium-derived NO synthesis is prevented by L-NAME. This compound is an analog of L-arginine that can not be metabolized and acts as a competitive inhibitor of NO synthase to block NO production. The increase in ACh sensitivity with this compound is of the same order of magnitude as that observed with $\alpha\alpha$Hb plus DFO, particularly during the supposed recovery phase after treatment. This delayed response appears to be consistent with previous findings [13] that L-NAME causes long-lasting increases in the coronary pressure of isolated rabbit hearts, presumably in part due to the lengthy time required to replace the major pool of intracellular L-arginine.

There have been numerous reports suggesting that NO, as the putative EDRF, has a significant role in regulating coronary vascular tone [13-15], and suppression of basal NO release has been associated with vasoconstriction in the coronary vascular bed of the isolated rabbit heart [13,16]. Other observers have demonstrated that hemoglobin may have a potent inhibitory effect on the vasodilation mediated by EDRF *in vitro* because of the extremely high affinity of its heme moiety for NO [6,17]. Although a parallelism between the formation of NO and ACh-induced coronary vasodilation has been reported [18], it has also pointed out that ACh has two distinct and opposite actions on blood vessels: direct constriction on muscarinic receptors of vascular smooth muscle and an indirect vasodilator action that is mediated by endothelium [19]. In our experiments, the impairment by $\alpha\alpha$Hb of endothelium-dependent NO-mediated vasodilation appears to have potentiated the coronary vasoconstriction induced by direct cholinergic stimulation of vascular smooth muscle with ACh. The lack of complete reversibility of this response after washout of the hemoglobin bolsters the argument that another mechanism is acting besides NO-binding by hemoglobin.

The decrease in sensitivity to ACh of our hemoglobin-treated preparations in the presence of DFO and the reversibility of the response when this iron chelator is included suggests that generation of cytotoxic oxygen-derived free radicals may be the most likely candidate for this additional mechanism. This is not inconsistent with previous suggestions that cell-free hemoglobin preparations promote such free radical formation during the process of autoxidation [9,20]. The release of iron from the heme pocket or the presence of adventitious iron contaminants might further catalyze the formation of both superoxide and hydroxyl radicals in the vascular endothelium, damaging cells and altering vascular tone [21,22]. The involvement of a cytotoxic component to these responses is further supported by the better myocardial performance displayed at the end of the experimental protocol in hearts perfused with $\alpha\alpha$Hb plus DFO. An additional possibility that must be considered is that heme transfer to vascular tissue might also interfere with the normal capacity of these cells to control vascular smooth muscle tone.

In summary, our study indicates that hemoglobin can interfere with normal steady-state vascular control mechanisms and suggests that these effects may be due to two simultaneous acting mechanisms involving inactivation of EDRF and iron-mediated generation of cytotoxic oxygen-derived free radicals. These two potentially toxic effects should be carefully evaluated in assessing safety of potential hemoglobin-based blood substitutes.

The opinions and assertions contained herein are the private views of authors and are not to be constructed as official, nor do they reflect the views of the Department of the Army or the Department of Defense (AR 360-5).

The experimental studies the authors described in this report were reviewed and approved by the Institutional Review Committee/Animal Care and Use Committed at Letterman Army Institute of Research. The

manuscript was peer reviewed for compliance before submission for publication. In conducting the research described here, the authors adhered to the "Guide for the Care and Use of Laboratory Animals" (NIH Pub. 85-23).

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574   MACDONALD AND MOTTERLINI

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PREVENTION OF SIDE EFFECTS BY HEMOGLOBIN SOLUTIONS;
THE SELECTION OF OPTIMAL TEST MODELS, ESPECIALLY
CONCERNING THROMBOGENICITY.

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ABSTRACT

Modification of hemoglobin (Hb) by crosslinking and polymerization results
in an improved oxygen release capacity and a prolonged vascular retention
time. Modification improves the efficacy and prevents certain side effects.
It eliminates leakage of Hb through the kidneys and accumulation in the tubuli.
Another important issue is the degree of purification of Hb solutions. Traces
of membrane fragments may cause immunogenic and thrombogenic side effects.
To determine the contamination with erythrocyte membrane fragments, we
developed assays for glycophorin-α and phospholipids. Special models were
evaluated for testing the maximum allowable level of membrane contamination.
As an in vitro model for thrombogenicity we used confluent monolayers of

577

human umbilical vein endothelial cells. These cells were incubated with Hb solutions and subsequently tested on tissue factor (TF) procoagulant activity. TF was tested by the factor VII-catalyzed activation of factor X. The lower detection limit of this assay for endotoxin was 0.5 ng/ml. Hb did not cause any tissue factor expression even after prolonged incubation. No cooperation was found within endotoxin.

As an in vivo test on thrombogenicity we developed a guinea pig model in which we can follow the generation of fibrinopeptide A (FPA). This is one of the most sensitive markers for thrombin activation in vivo. When slightly contaminated Hb solutions (phospholipid content 2 nmol/ml) were infused in the presence of factor Xa at a dose (9 μg/kg) which in itself did not induce FPA generation, we observed an increase in FPA levels in the plasma from 1.2 $\pm$ 0.4 ng/ml to 5.2 $\pm$ 0.7 ng/ml. Factor Xa is used to mimic a stressed clinical condition with activated coagulation. We conclude that this stressed animal model is most suitable for the estimation of thrombogenic risks of transfusions with Hb solutions.

INTRODUCTION

In several cases, infusion of hemoglobin preparations has been found to elicit procoagulant responses. The thrombogenic effects observed have been ascribed to the presence of contaminating endotoxins or to incomplete removal of red blood cell stromal components (1). On the other hand, infusion of stroma free hemoglobin has been demonstrated to cause disseminated intravascular coagulation (2). Therefore, one cannot rule out the possibility that hemoglobin itself, apart from its contaminants, contributes to the thrombogenic risk

associated with its application. In the present study, an effort was made to determine if vascular endothelium participates in the thrombogenic response towards hemoglobin solutions. Vascular endothelial cells play a central role in the regulation of haemostasis and are among the first cells in line to become exposed to eventual toxic side effects of hemoglobin solutions. It is therefore not unthinkable that the procoagulant responses observed following infusion of hemoglobin are, to a certain extent, caused by impairment of vital endothelial cell functions. One of the most critical endothelial cell functions with respect to its regulatory role in haemostasis is the control of tissue factor expression levels. Stimulation of endothelial cells with endotoxins or interleukin-1 readily results in cell surface expression of tissue factor. Exposition of tissue factor to the blood stream is regarded as the major trigger of blood coagulation in vivo. These considerations have led us to evaluate tissue factor procoagulant activity on vascular endothelial cells following their incubation with hemoglobin solutions.

As an in vivo test on thrombogenicity we developed a guinea pig model in which we can follow fibrinopeptide A (FPA) generation using an immunoassay. This is one of the most sensitive markers for thrombin activation in vivo. With these models the following questions were addressed:

1) Is Hb in itself thrombogenic?

2) Which model is suitable to exclude thrombogenic side effects of hemoglobin solutions under different clinical conditions?

METHODS

1) In vitro model for thrombogenicity: <u>Endothelial cell culture.</u>

-Confluent monolayers of human umbilical vein endothelial cells were incubated

with Hb solutions or red blood cell lysates and/or endotoxin for a time period
of 30 minutes to 24 hours.

Incubation with only the 10% serum containing medium served as control.

After incubation the cells were tested on tissue factor (TF) procoagulant activity
as follows:

-Cells were washed with Tris-buffer (pH 7.4) which was supplemented with
0.5 mM EDTA and 5 mg/ml ovalbumin during the second wash step.

-Subsequently, the cells were preincubated with factor VIIa before factor X
was added.

-Formation of factor Xa was monitored by measuring its amidolytic activity
towards the chromogenic substrate S2337 in subsamples drawn every 5 minutes
after factor X addition. Initial factor Xa activation rates (vi) were calculated
from the linear increase in factor Xa measured with time and served as a
measure for TF-dependent procoagulant activity.

2) In vivo model for thrombogenicity: Guinea pig model.

In this model we follow the generation of fibrinopeptide A (FPA). This is
one of the most sensitive markers for thrombin activation in vivo.

For full details, see Biessels et al., : "Fibrinopeptide A generation in the guinea
pig as an in vivo model for measuring coagulation effects of hemoglobin
solutions".

RESULTS

The effect of stroma free hemoglobin on induction of tissue factor procoagulant
activity by LPS is shown in Figure 1.

-The figure shows a dose-dependent effect of LPS on tissue factor procoagulant
activity of endothelial cells
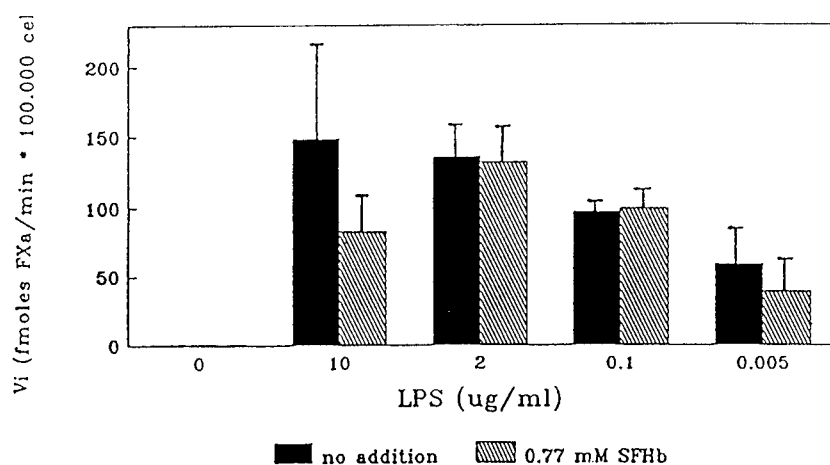
Figure 1.

Confluent endothelial cell monolayers were cultured for 4 hours with LPS either in the absence or in the presence of 0.77 mM stroma free hemoglobin (SFHb). Subsequently cell monolayers were washed and assayed for tissue factor procoagulant activity as described in the Methods. Data are the mean +/- SEM of three independent experiments.

-LPS concentrations as low as 0.5 ng/ml still cause detectable tissue factor activity on the endothelium.

-Addition of stroma free hemoglobin alone did not cause any detectable expression of tissue factor procoagulant activity on the endothelium.

-Tissue factor activity induction by LPS was hardly affected by the presence of stroma free hemoglobin.

The effect of incubation times, LPS contamination and stroma contamination of hemoglobin on the induction of endothelial procoagulant activity is shown in Figure 2.

-Following a 4 hour incubation period with SFHb solutions, endothelial cells did not show any tissue factor procoagulant activity.

Vi (fmoles FXa/min * 100.000 cells)
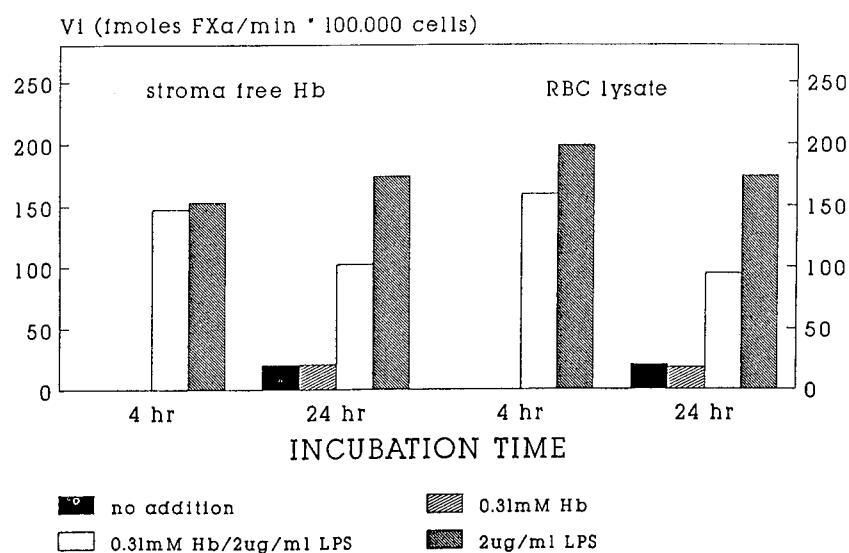
Figure 2.

Confluent endothelial cell monolayers were cultured for 4 hours or 24 hours with stroma free hemoglobin (SFHb) or red blood cell (RBC) lysates (Hb concentration: 0.31 mM). When indicated, LPS (2 ug/ml) was present during the last 4 hours of incubation. After incubation, cell monolayers were washed and assayed for tissue factor procoagulant activity as described in the Methods. Data are the mean of 3 or 2 experiments.

-When the hemoglobin incubation period was extended to 24 hours the tissue factor procoagulant activity detected did not exceed control levels.

-No cooperativity could be observed between LPS and hemoglobin in triggering tissue factor expression (Figs. 1 and 2).

-Incubation of the endothelial cell monolayers with red blood cell lysates gave similar results as with stroma free hemoglobin incubations (Fig. 2).

The guinea pig model:

Figure 3 shows the FPA levels in guinea pigs after 30% exchange transfusion.

FPA generation after
a 30% exchange transfusion
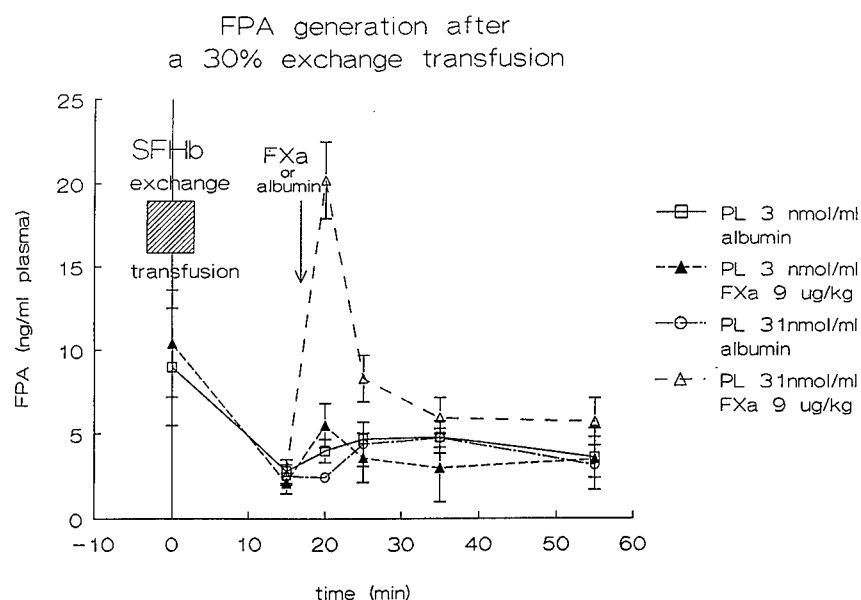
Figure 3.

FPA levels in guinea pigs after 30% exchange transfusion with SFHb.

-15 minutes after exchange transfusion with SFHb FPA levels returned to basal values.

-16 minutes after exchange transfusion albumin or Factor Xa was infused in the presence of phospholipid (PL).

-the combination of Factor Xa (9ug/kg) and phospholipid (31 nmol/ml) gave a substantial rise in FPA levels.

CONCLUSIONS

From the endothelial cell model:

1) Hb does not induce TF-activity, even after prolonged incubation (up to 24 hours).

2) RBC stroma does not induce TF activity.

3) Hb does not potentiate the induction of TF activity by LPS.

These observations are inconsistent with those of Feola et al. (3) who have reported that vascular endothelium exposed to hemoglobin solutions containing stromal aminophospholipids are triggered to express tissue factor procoagulant activity. However, Feola et al. measured tissue factor activity in the incubation supernates of hemoglobin exposed cells by means of a one stage clotting assay. Consequently, the results of Feola et al. fail to prove that the procoagulant responses observed following incubation of endothelium with stroma contaminated hemoglobin is caused by tissue factor expression. Our data suggest that the procoagulant responses towards infusion of either stroma free or stroma contaminated hemoglobin solutions do not include a direct provocation of tissue factor expression on vascular endothelium.

from the guinea-pig model;

1) Pure hemoglobin gives no FPA generation.

2) After exchange transfusion with a Hb solution there is -in the presence of factor Xa- a correlation between phospholipid contamination and in vivo FPA generation.

general conclusions;

1) The guinea-pig model can be considered as a stressed animal model mimicking a clinical condition under which the coagulation system is activated.

2) The guinea pig will be useful to define the maximal allowable level of thrombogenic factors like phospholipid contamination.

Both models give no support for the suggestion that Hb itself induces procoagulant activity as concluded by Smith and Winslow (4) on the basis of their in vitro observations with mononuclear cells from peripheral blood.

ACKNOWLEDGEMENTS

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DIASPIRIN CROSSLINKED HEMOGLOBIN: EVALUATION OF EFFECTS ON THE MICROCIRCULATION OF STRIATED MUSCLE

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ABSTRACT

Hemoglobin-based oxygen carriers such as diaspirin-crosslinked hemoglobin (DCLHb) have been proposed for blood substitution due to their plasma expansion and oxygen transport capacity. This study investigates the effects of DCLHb on the microcirculation of striated muscle after moderate topload infusion and isovolemic exchange transfusion in awake hamsters.

The skinfold chamber model in hamsters and intravital fluorescence microscopy were used for analysis of vessel diameter, red blood cell velocity (RBCV), leukocyte sticking to the microvascular endothelium, and macromolecular leakage in striated skin muscle. In each animal, arteriolar and postcapillary vessel segments were chosen and sequentially recorded on videotape (baseline). Animals were subjected to either topload infusion (10% of blood volume) or isovolemic exchange transfusion (hct 30%) of DCLHb followed by measurements at 10, 30, and 60 min thereafter. In vivo visualization of plasma and leukocytes was performed using FITC-dextran 150.000 and rhodamine 6G, respectively.

No significant changes of vessel diameter and RBCV were observed after topload infusion or isovolemic exchange transfusion with DCLHb, either in postcapillary venules or in arterioles when compared with baseline values. Leukocyte sticking and macromolecular leakage were not found enhanced after administration of DCLHb.

We conclude that the introduction of DCLHb-bound oxygen into the tissue does neither stimulate leukocyte adhesion nor impair endothelial integrity.

587

INTRODUCTION

Many efforts have been made to develop a hemoglobin-based oxygen carrier (HBOC, for nomenclature see [1]) that could serve as universal plasma expander and oxygen carrier. However, the breakthrough of HBOCs into clinical use has not yet occurred because of the toxic potential of stroma-free hemoglobin per se and/or stromal contaminants [2,3]. The chemical modification of the hemoglobin molecule by covalent crosslinking of the α-chains [4,5] has been shown to be free of nephrotoxic side effects. These findings have been attributed to the inhibition of the dissociation of the hemoglobin tetramer into $\alpha\beta$-heterodimers, leading to acute renal failure through obstruction of the kidney's tubular system by the filtered $\alpha\beta$-heterodimers.

Despite the successful management of renal toxicity, there is still major concern on the vasoconstricting potential of hemoglobin solutions [6]. The underlying mechanisms of the vasoactive side effects are still unknown. It has been suggested that free hemoglobin may act as a scavenger of endothelial derived relaxing factor (EDRF) [7] or via release of endothelin-1 [8], accounting for the observed vasoconstricting potential of hemoglobin.

Since direct *in vivo* visualization is required for investigation of the vasoactive effects of hemoglobin solutions, we assessed the effects of diaspirin crosslinked hemoglobin (DCLHb) on the microvasculature of striated skin muscle in hamsters using intravital fluorescence microscopy.

MATERIALS AND METHODS

Animal model. Fluorescence microscopy and the dorsal skinfold chamber model in awake Syrian golden hamsters were used for intravital microscopic quantification of vessel diameter, red blood cell velocity, leukocyte-endothelium interaction, and macromolecular leakage of FITC-dextran 150,000 in the striated skin muscle (for technical details see [9-11]).

Intravital fluorescence microscopy. A 25-fold water immersion objective (total magnification 560-fold) was used for investigation of arterioles, postcapillary venules and capillaries. By means of a computer-controlled stepping motor, the identical vessel segments and regions of interest can be recalled at the preset time points of

investigation. The microscopic images were recorded on video-tape and analyzed off-line with respect to red blood cell velocity (mm/s), vessel diameter (μm), leukocyte-endothelium interaction, and macromolecular leakage of fluorescein-isothiocyanate-labelled (FITC) dextran 150,000 (5 mg in 0.1 ml saline, Pharmacia, Sweden) using a computer-assisted microcirculation analysis system (CAMAS, [12]). Leukocyte sticking to the microvascular endothelium was assessed after in vivo staining of white blood cells with rhodamine 6G (0.05 mg in 0.1 ml saline, Sigma Chemical Company, Germany). Sticking leukocytes were defined as cells which did not detach from the endothelial lining within an observation period of one minute and are given as number per mm^2 endothelial surface (cells/mm^2). Macromolecular leakage was assessed by densitometric quantification of the fluorescence intensity where the plasma marker FITC dextran had extravasated into the perivascular tissue (in % change of baseline).

Experimental Design. Animals (n=5 per experimental group) received either a 10% topload infusion (10% of total blood volume) of diaspirin crosslinked hemoglobin (DCLHb, Baxter Healthcare Corp., Illinois, USA), or were isovolemically exchange-transfused by simultaneous blood withdrawal from the carotid artery and infusion of DCLHb via jugular vein to a hematocrit of 30 ± 5 % as previously described [13]. The microcirculatory parameters were assessed prior to topload or exchange transfusion and 10, 30 and 60 minutes thereafter.

Hemoglobin solution. Diaspirin crosslinked hemoglobin was provided by Baxter Healthcare Corporation, Deerfield, Illinois, USA (for detail see table 1).

RESULTS

Topload infusion of DCLHb did not elicit significant changes of the microhemodynamic parameters vessel diameter and red blood cell velocity, either in arterioles (data not shown) or in postcapillary venules, when compared with baseline values. Likewise, leukocyte sticking was not found enhanced after topload infusion which was accompanied by the absence of macromolecular leakage of FITC dextran (table 2a).

Isovolemic exchange transfusion of ~ 50 % of total blood volume with DCLHb (35 ± 5 ml kg^{-1} b.w., hematocrit 30 %) was well tolerated by the animals. After administration of this dose, the microcirculatory parameters remained unchanged when compared with pre-exchange transfusion baseline values (table 2b).

TABLE 1.   **Properties of diaspirin crosslinked hemoglobin (DCLHb)**

Hemoglobin concentration	13.8 g%	pH at 25°C	7.75
Methemoglobin	7.6%	P_{50}	34.0 mmHg
Sodium	132.0 mval	Oncotic pressure	58.0 mm Hg
Potassium	4.0 mval	Endotoxin	< 0.50 U / ml
Osmolarity	284.0 mosm/L		

TABLE 2. *Microcirculatory effects of DCLHb on the microcirculation of striated muscle after moderate topload infusion and isovolemic exchange transfusion.* Depicted are microcirculatory parameters assessed in 6 - 8 postcapillary venules ($\varnothing$: 20-45 µm) investigated in each animal. Macromolecular leakage is given in % change of baseline. Data are mean ± SD of 5 animals per experimental group.

Treatment	Parameter	Baseline	Time after treatment		
			10 min	30 min	60 min
a. Topload	Diameter [µm]	27 ± 6	27 ± 6	27 ± 6	28 ± 5
	RBCV [mm/s]	0.4 ± 0.2	0.5 ± 0.3	0.6 ± 0.2	0.4 ± 0.3
	Sticking Leukocytes [cells/mm^2]	40 ± 51	44 ± 53	31 ± 41	25 ± 34
	Macromolecular Leakage [Δ%]	—	10 ± 18	9 ± 16	1 ± 15
b. Isovol. Exchange Transfusion	Diameter [µm]	26 ± 5	28 ± 14	28 ± 6	27 ± 6
	RBCV [mm/s]	0.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.2	0.3 ± 0.3
	Sticking Leukocytes [cells/mm^2]	35 ± 16	46 ± 26	30 ± 54	33 ± 46
	Macromolecular Leakage [Δ%]	—	5 ± 18	17 ± 11	18 ± 15

DISCUSSION

In the present study, first in vivo microcirculatory analysis was performed to determine the effects of the hemoglobin-based oxygen carrier diaspirin crosslinked hemoglobin (DCLHb, [4,5]) on the microvasculature of striated muscle. We have shown that no adverse effects on both the *micro*hemodynamics of striated muscle perfusion and leukocyte-endothelium interaction are to be expected after both topload infusion and isovolemic exchange transfusion by DCLHb.

The hamster skinfold chamber model has proven as a valid model for the investigation of the microcirculation in the fine striated skin muscle of awake hamsters [10,11,13-16]. Due to the integrity of the microvasculature and the opportunity of chronic observation, this model appears particularly suitable for the investigation of endothelium-dependent and -independent changes of vessel diameter.

The lack of vasoconstricting effects of DCLHb on both arterioles and postcapillary venules suggests that α-α crosslinked human hemoglobin does not affect the physiologic balance of vasoconstricting and vasodilating mechanisms. We therefore conclude that under our experimental conditions, DCLHb exerts no biologically significant action on endothelium-derived constricting factors, as suggested by others [7]. We furthermore found that the integrity of the endothelium of arterioles and postcapillary venules of striated muscle was not altered after infusion of DCLHb, as indicated through the lack of leakage of FITC-dextran 150,000 into the perivascular space.

The absence of enhanced leukocyte sticking within the identical vessel segments (table 2), representing a highly sensitive indicator of the early inflammatory reaction to noxious stimuli [17], suggests that an oxygen radical dependent activation of leukocytes, potentially initiated by the release of hemoglobin-bound iron ions [18], is unlikely to occur under the experimental conditions of this study.

Whether these findings hold also true for pathophysiological situations such as ischemia-reperfusion and shock, remains to be investigated in future studies.

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REGIONAL CIRCULATORY AND SYSTEMIC HEMODYNAMIC EFFECTS OF
DIASPIRIN CROSS-LINKED HEMOGLOBIN IN THE RAT

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ABSTRACT

Diaspirin cross-linked hemoglobin (DCLHb™) (Baxter Healthcare Corporation) is a promising resuscitative fluid. The effect of DCLHb (400 mg/kg, iv), on regional circulation and systemic hemodynamics was studied in male Sprague-Dawley rats using a radioactive microsphere technique. Systemic hemodynamics, distribution of cardiac output, regional blood flow and vascular resistance were determined before (baseline) and 15, 30 and 60 min after the administration of DCLHb. Infusion of an equal volume of saline did not produce any significant change in systemic hemodynamics or regional circulation. DCLHb produced an increase (79%) in the mean blood pressure which lasted for more than 60 min. Heart rate, cardiac output and stroke volume were not significantly affected, while total peripheral resistance was increased after the administration of DCLHb. DCLHb produced significant increases in blood flow to the heart, gastrointestinal tract (GIT), portal system and skin. The blood flow to kidney, brain and musculoskeletal system was not significantly affected by DCLHb. The vascular resistance was not altered in the heart, brain, GIT, portal system, kidney or skin, but there was a marked increase in the vascular resistance in the musculoskeletal system. There was a significant increase in the percentage of cardiac output to visceral organs like heart, GIT and portal system, while a marked decrease in the percent cardiac output to musculoskeletal system was observed with DCLHb. It is concluded that the blood flow to most of the organs is either increased or is not affected by DCLHb.

INTRODUCTION

There has been tremendous progress in the development of hemoglobin solutions as resuscitative solutions. Several investigators have stressed a number of advantages of hemoglobin solutions over other resuscitation solutions (1-4).

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[1]For correspondence

Diaspirin cross-linked hemoglobin (DCLHb) is a blood substitute derived from the human erythrocytes. DCLHb is produced by cross-linking molecular hemoglobin between the α-subunits by means of a reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (5). The cross-linking of the α subunits affords the hemoglobin a favorable oxygen dissociation curve (6,7). The manufacturing process also includes heat pasteurization of the solution, as described by Estep et al. (8,9). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (5).

DCLHb has been found to be an effective resuscitative fluid following hemorrhage (10). It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions are not affected significantly (11). DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters (12) in a rodent hemorrhage model. DCLHb has also been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion (13) in rats.

In normal adult rats DCLHb has been found to increase the mean arterial blood pressure (14), while several other hemoglobin solutions have been reported to produce a rapid and sustained increase in mean arterial pressure (15-17). Whereas purified stroma-free hemoglobin solutions have been reported to produce vasoconstrictor activity in a variety of experimental models (18-20). Yamakawa et al. (21) reported that stroma-free hemoglobin solution produced marked vasodilatation of coronary blood vessels, when administered to the rat. However, the effect of DCLHb on the regional blood circulation has not been studied. The present study was conducted to determine the effect of DCLHb on regional blood flow and systemic hemodynamics in the rat.

METHODS

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 300-350 g were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683. The carotid artery of the right side was exposed and a PE 50 cannula guided through the common carotid artery to the left ventricle. The femoral artery of the right side was cannulated and the cannula guided to the abdominal aorta and connected to a withdrawal pump (Harvard Model 22).

At each measurement, a suspension of approximately 200,000 microspheres (15 $\pm$ 1 μm diameter) labeled with [46]Sc (Scandium), [113]Sn (Tin), [141]Ce (Cerium) or [103]Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA) in 0.2 ml saline were injected into the left ventricle after thoroughly mixing and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood

flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the catheter inserted in the abdominal aorta via the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: brain, kidneys, heart, gastrointestinal tract (stomach, small intestine, caecum, large intestine, mesentry and pancreas), liver, spleen, skin and the rest of the body consisting of muscles and bones. The radioactivity in the microspheres injected, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output (2) stroke volume (3) total peripheral resistance (4) regional blood flow and (5) regional vascular resistance. The data was calculated using the programs described by Saxena, et al. (22). Infusion of saline did not affect the systemic hemodynamics or regional circulation. The effect of DCLHb (400 mg/kg, iv) on systemic hemodynamics and regional circulation was studied. The dose of DCLHb was selected on the basis of the earlier studies conducted in several laboratories (14,23).

All data are presented as the mean values ± 1 SEM. Data were analyzed by analysis of variance followed by Duncan's or Scheffe's S test. A level of $P < 0.05$ was considered significant.

RESULTS

Effect of DCLHb on the systemic hemodynamics

DCLHb (400 mg/kg, i.v.) produced a significant [$F(3, 20) = 11.28$; $p = 0.008$) increase in the mean arterial blood pressure 15, 30 and 60 min after administration. The heart rate, cardiac output and stroke volume were not affected after DCLHb administration. DCLHb significantly increased the total peripheral resistance at 15 and 60 min [$F (3,20) = 2.72$; $p = 0.04$] (Table 1).

Effect of DCLHb on the regional blood flow

DCLHb (400 mg/kg, iv) significantly increased [$F (3,20) = 4.09$; $p = 0.026$] the blood flow to the heart, at 15, 30 and 60 min after infusion (Fig. 1). Blood flow was significantly increased to the GIT [$F (3,20) = 3.62$; $p = 0.022$] and portal system [$F (3,20) = 3.54$; $p = 0.03$] 15 and 30 min after DCLHb infusion and skin [$F (3,20) = 2.71$; $p = 0.03$] 30 min after the administration of DCLHb (Fig. 2). Blood flow to the brain, kidney and musculoskeletal system was not significantly affected by DCLHb (Fig. 2).

Effect of DCLHb on the regional vascular resistance

DCLHb did not affect the vascular resistance in the heart (Fig. 1) brain, GIT, portal system, kidney and skin (Fig. 3). The vascular resistance was found to be significantly increased in the musculoskeletal system [$F (3,20) = 5.57$; $p = 0.01$] at 15, 30 and 60 min after the administration of DCLHb (Fig. 3).

Effect of DCLHb on the distribution of cardiac output

The percent cardiac output was significantly increased to the heart [$F (3,20) = 10.44$; $p = 0.01$] at 15, 30 and 60 min after DCLHb infusion and the

SHARMA, REBELLO, AND GULATI

Table 1: Effect of DCLHb (400 mg/kg, iv) on the systemic hemodynamics in rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)	386 ± 10	397 ± 10	398.3 ± 11	395 ± 10
Mean B.P.(mmHg)	84 ± 6	150 ± 9*	144 ± 11*	125 ± 9*
Cardiac output (ml/min)	82 ± 10	107 ± 7	119 ± 20	86 ± 6
Stroke Volume (ml)	0.2 ± 0.02	0.3 ± 0.02	0.3 ± 0.06	0.2 ± 0.02
TPR (MBP/CO)	1002 ± 82	1493 ± 149*	1347 ± 207	1570 ± 110*

*p < 0.05 as compared to control; N = 6.
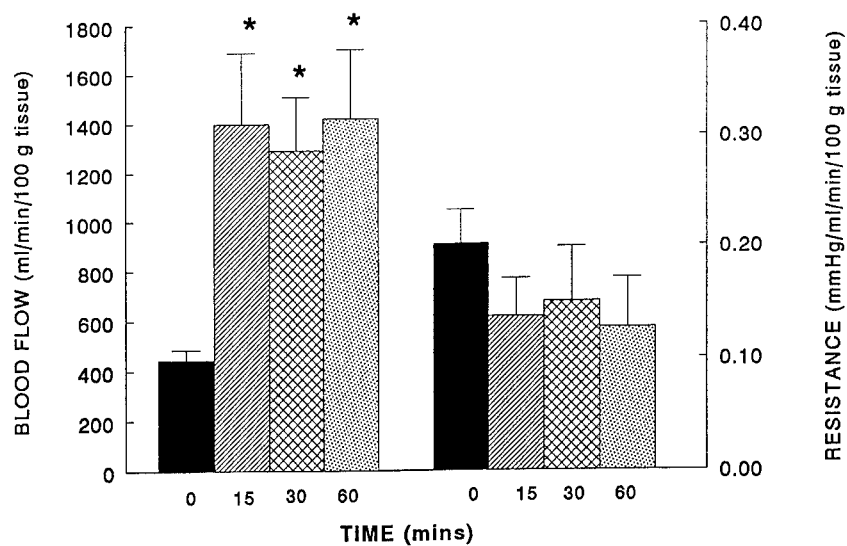
Fig. 1 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow to the heart
(ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g
tissue) before (0) and at 15 min, 30 min and 60 min after its
administration to the rats. Asterisks indicate significant difference as
compared to control.
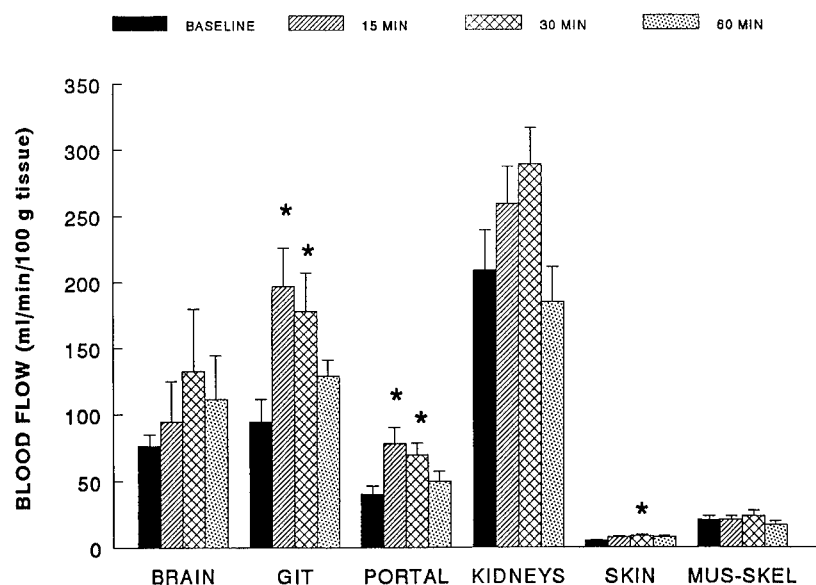
Fig. 2 The effect of DCLHb (400 mg/kg, i.v.) on the regional blood flow (ml/min/100 g tissue) before (0) and at 15 min, 30 min and 60 min after its administration to the rats. Asterisks indicate significant difference as compared to control.

portal system [F (3,20) = 4.05; p = 0.02] and GIT [F (3,20) = 2.72; p = 0.04] 15 min after infusion. On the other hand, the percent cardiac output to the musculoskeletal system was found to be significantly decreased [F (3,20) = 5.39; p = 0.01] 15, 30 and 60 min after DCLHb administration. The percent cardiac output to the brain, kidney and skin was not altered by DCLHb administration (Fig. 4).

DISCUSSION

Stroma-free hemoglobin solutions have been reported to produce a pressor effect (12,15-17,24). DCLHb has also been demonstrated to produce a pressor response in rats (14,24). In the present study DCLHb was found to produce a marked increase in the mean arterial pressure and total peripheral resistance in rats. Cardiac output, stroke volume and heart rate were not altered. The blood flow to the heart, GIT, portal system and skin increased while it was not altered in brain, kidney or the musculoskeletal system. The vascular resistance was not affected in most of the organs except in the musculoskeletal system in which a marked increase was observed. It appears
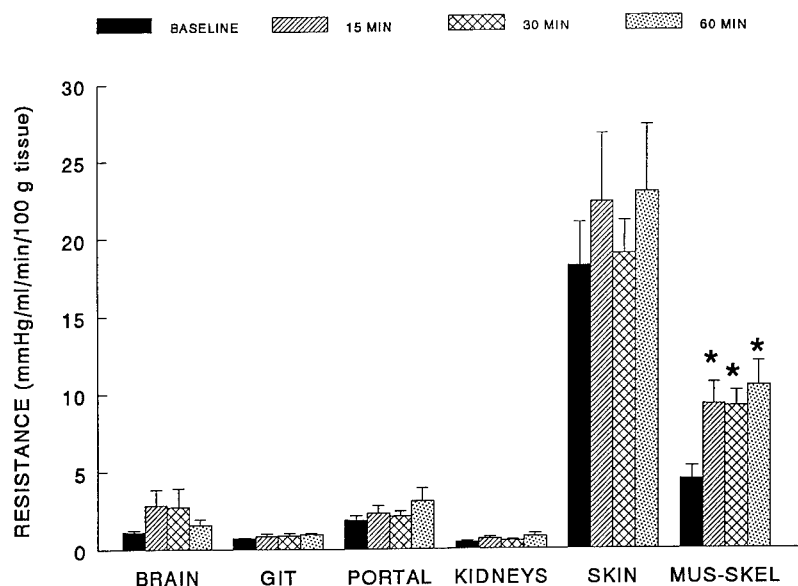
Fig. 3 The effect of DCLHb (400 mg/kg, i.v.) on   the regional vascular
       resistance (mmHg/ml/min/100 g tissue) before (0) and at 15 min, 30 min
       and 60 min after its administration in rats. Asterisks indicate significant
       difference as compared to control.

that the blood flow to most of the visceral organs is increased, probably due to
the redistribution of blood from the musculoskeletal system.   This was
confirmed by determining the percent cardiac output to various organs systems.
It was found that the percent cardiac output to heart, GIT and portal system is
increased while it is significantly decreased to the musculoskeletal system.
Since the major portion (55%) of the cardiac output goes to the
musculoskeletal system, a small decrease in percent cardiac output to the
musculoskeletal system could lead to a significant increase in the blood flow to
other organs. It is concluded that DCLHb increases the blood flow to several
visceral organs due to the redistribution of blood flow from the musculoskeletal
system.

      Hemoglobin solutions or red cell lysates have been shown to possess
vasoconstrictor actions on various types of arteries in different animals
(20,21,25). However, it is not clear whether it is the hemoglobin molecule or
some other associated factors responsible for the vasoconstrictor effect. The
vasoconstriction induced by hemoglobin has been attributed to the hemoglobin
molecule or a very closely associated contaminant (19,20).
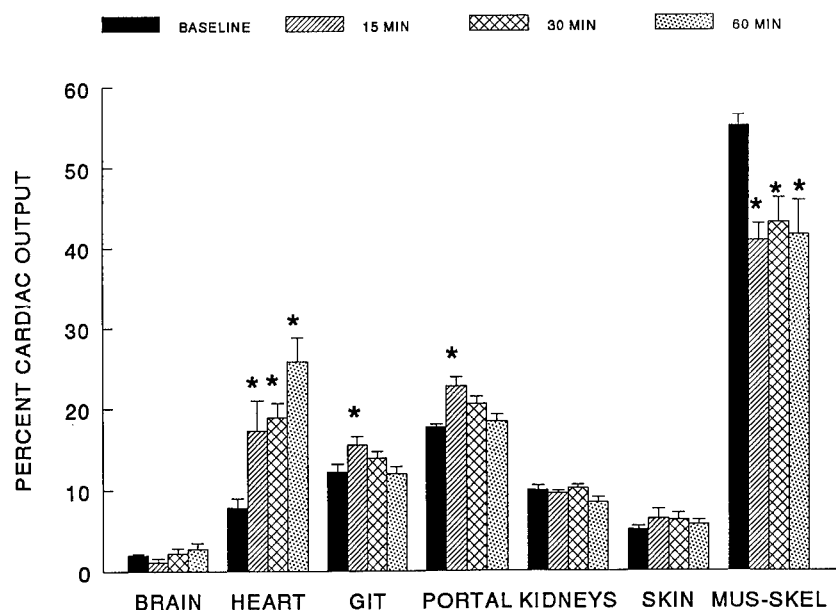
Fig. 4 The effect of DCLHb (400 mg/kg, i.v.) on the percent cardiac output to various tissues before (0) and at 15 min, 30 min and 60 min after its administration to the rats. Asterisks indicate significant difference as compared to control.

Studies have demonstrated that chemical modifications of hemoglobin by conversion to met-hemoglobin (19) or by glutaraldehyde cross-linking (19,26) can significantly reduce the constrictor activity of hemoglobin. The vasoconstrictor activity is reduced in hemoglobin solutions purified by affinity chromatography or preparative HPLC (27) but not by membrane dialysis or ultrafiltration (19). Removal of stromal fraction has been demonstrated to diminish vasoconstrictor action in isolated rabbit heart (28). MacDonald et al. (29) found that cross-linked hemoglobin solution has less vasoconstrictor activity as compared with unmodified hemoglobin on coronary blood vessels. Lieberthal et al. (30) also found that cross-linked hemoglobin solution did not while unmodified hemoglobin solution significantly increased the perfusion pressure and the resistance of the coronary blood vessels. These studies suggested that besides chemical modification, the stromal fraction could be contributing towards vasoconstrictor activity.

DCLHb is a chemically modified (intramolecular cross-linking of α subunits) and highly purified (including heat pasteurization) hemoglobin solution. The present study using an in vivo model shows that the blood flow to most of the organs is either increased or is not affected by administration of DCLHb.

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DIASPIRIN CROSS-LINKED HEMOGLOBIN (DCLHB™): INVOLVEMENT OF ADRENERGIC MECHANISMS IN THE PRESSOR EFFECT

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ABSTRACT

Diaspirin cross-linked Hemoglobin (DCLHb™) (400 mg/kg, i.v.), a resuscitative solution, produces a pressor effect in rats and several other species. Studies were conducted to determine the role of the central nervous system and adrenal medulla in the pressor effect of DCLHb in rats. Intravenous administration of DCLHb produced an increase in blood pressure in cervical sectioned animals, which was comparable to that observed in normal rats. This indicates that the pressor effect of DCLHb was mediated through the peripheral vascular system rather than through the central nervous system. DCLHb produced a pressor effect in bilateral adrenal demedullated rats that was similar to normal rats, suggesting that the pressor effect is not through the release of catecholamines or other pressor substance from the adrenal medulla. The effects of DCLHb pretreatment on norepinephrine (0.5 μg/kg), phenylephrine (5 μg/kg) and clonidine induced blood pressure and heart rate responses were also studied. DCLHb significantly potentiated the pressor response to norepinephrine and phenylephrine. Clonidine normally produces a fall in blood pressure by acting on the central α-adrenoceptors, and a rise in blood pressure by stimulating the peripheral vascular α-adrenoceptors. DCLHb produced a marked potentiation of the pressor response to clonidine (75 μg/kg, i.v.), that masked the central depressor effect. The specificity of the potentiation was confirmed by using phenoxybenzamine, prazosin, and yohimbine. In order to exclude the contribution of a centrally induced cardiovascular effect of clonidine, further studies were carried out in cervical sectioned rats. DCLHb markedly potentiated the pressor effect of clonidine (25 μg/kg, i.v.) in cervical sectioned rats. This potentiation could be attenuated by prazosin and yohimbine. It appears that in rats α-adrenoceptors in the peripheral vascular system are sensitized by DCLHb.

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603

INTRODUCTION

DCLHb, a blood substitute derived from human erythrocytes, is prepared by cross-linking purified hemoglobin between the α-subunits with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (1). The crosslinked product is heat treated in solution, as described by Estep et al. (2,3), to inactivate any contaminating viruses and precipitate undesired proteins. DCLHb was found to be a promising resuscitative fluid after hemorrhage (4). Resuscitation with DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters (5). In addition, DCLHb has been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion (6) in rats.

Hemoglobin solutions also have been reported to produce a rapid and sustained increase in mean arterial pressure (7,8,9). DCLHb increases the mean arterial pressure when administered in hemorrhagic rats (5) and in normal adult rats (10). The mechanism(s) responsible for the increase in blood pressure following DCLHb administration remain to be elucidated.

The pressor effect of DCLHb could be due to interaction with the vasomotor nuclei in the central nervous system, the sympathetic system or by action on the peripheral vascular system. A modification in one or more of several factors regulating blood pressure, such as EDRF/NO, endothelin, renin-angiotensin, or adrenergic agents may be responsible for this pressor effect. The present study attempts to elucidate the role of adrenergic mechanisms in the pressor effect of DCLHb.

METHODS

Animals

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 300-350 g were housed three to a cage, in a room with controlled temperature (23 $\pm$ 1 °C), humidity (50 $\pm$ 10%) and artificial light (0600-1800 hr). The animals were given food and water ad libitum. The experiments were begun only after the animals were acclimatized to the environment.

Drugs

Clonidine, norepinephrine, phenylephrine, yohimbine and prazosin were purchased from Sigma Chemical Co., St. Louis, MO. Phenoxybenzamine was obtained from Smith Kline and French Labs., Philadelphia, PA. Diaspirin cross-linked hemoglobin in lactated electrolyte was provided by Baxter Healthcare Corporation, Round Lake, IL. All drugs were prepared in normal saline, except yohimbine and prazosin, which were first dissolved in ethanol and propylene glycol, respectively, and then diluted with normal saline. The drugs were prepared fresh at the time of each experiment.

Determination of blood pressure and heart rate

The animals were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 polyethylene tubing) for drug administration. The left femoral artery was cannulated (PE 50 polyethylene tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was

recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO2, pCO2 and pH constant, and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683.

Cervical section
Rats were prepared as described above for measurement of blood pressure and heart rate. A dorsal midline incision was made and the vertebral muscles were separated and retracted to expose the occipital bone and cervical vertebrae. The spinous process and lamina of upper cervical vertebrae were dissected out and removed to expose the spinal cord. The spinal cord was sectioned in the rats at the cervical level and a part of the cervical spinal cord was removed so that there was absolutely no connection with the brain. Animals were stabilized for one hour after which drugs were administered.

Bilateral adrenal demedullation
The bilateral adrenal demedullation was performed as described by Borkowski and Quinn (11) and subsequently by Gulati and Bhargava (12). Briefly, the animals were anesthetized with sodium pentobarbitone (40 mg/kg, i.p.) and a single midline incision was made in the abdomen. The adrenal gland of one side was exposed, a small incision was made in the cortex and the medulla was enucleated by gently squeezing the gland with the help of a small blunt forcep. Similarly, the adrenal medulla on the other side was removed. The abdomen was closed in layers. In a separate set of experiments, 6-8 rats were subjected to the same procedure except that their adrenals were not enucleated. These animals served as the sham treated group. Throughout the surgical procedure strict aseptic conditions were used. After a recovery period of seven days the rats were used for experiments.

Statistics
All data are presented as the mean values ± 1 SEM. The systolic pressure and heart rate were recorded as mmHg and beats/min, respectively. Data were analyzed by analysis of variance followed by Scheffe's S test. A level of $P < 0.05$ was considered significant.

RESULTS

Effect of cervical section or bilateral adrenal demedullation on the pressor effect of DCLHb
In intact and cervical sectioned rats, DCLHb (400 mg/kg, i.v.) was found to produce a similar increase in systolic blood pressure (Fig. 1). The increase in systolic pressure induced by DCLHb (400 mg/kg, i.v.) in bilateral adrenal demedullated rats was also found to be similar to that observed in sham treated rats (Fig. 2).

Effect of DCLHb pretreatment on adrenergic responses
Norepinephrine (0.5 μg/kg, i.v.) produced an increase in systolic pressure and a reflex bradycardia. DCLHb pretreatment produced a significant ($F_{(2,18)}$ = 22.84; $P = 0.001$) potentiation of the pressor effect of norepinephrine. In order
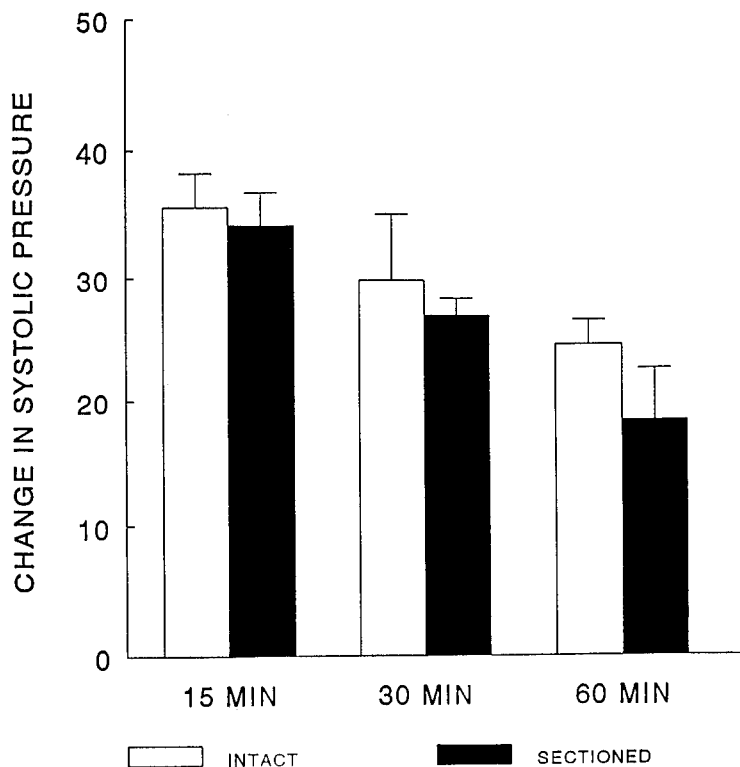
Fig. 1 The effect of DCLHb (400 mg/kg, i.v.) on the change in systolic blood
         pressure (mmHg) in intact (blank bars) and cervical sectioned (filled bars)
         rats.

to determine the specificity of this potentiation, pretreatment was also performed
with phenoxybenzamine (5 mg/kg, i.v.) prior to the administration of DCLHb and
norepinephrine. It was observed that the potentiation of the pressor effect of
norepinephrine by DCLHb was completely blocked $(F(2,18) = 22.84; P < 0.0005)$
by phenoxybenzamine (Figs. 3 and 4).

Phenylephrine (5 μg/kg, iv) produced an increase in systolic pressure
accompanied by a reflex bradycardia. DCLHb pretreatment significantly potentiated
the increase in systolic pressure $(F(2,20) = 28.80; P = 0.017)$ and decrease in
heart rate $(F(2,20) = 57.47; P < 0.0001)$ induced by phenylephrine. Prazosin (1
mg/kg, iv) pretreatment prior to the administration of DCLHb and phenylephrine
completely antagonized the effect on systolic pressure $(F(2,20) = 28.80; P <
0.0005)$ and heart rate $(F(2,20) = 57.47; P < 0.0001)$ (Figs. 3 and 4).
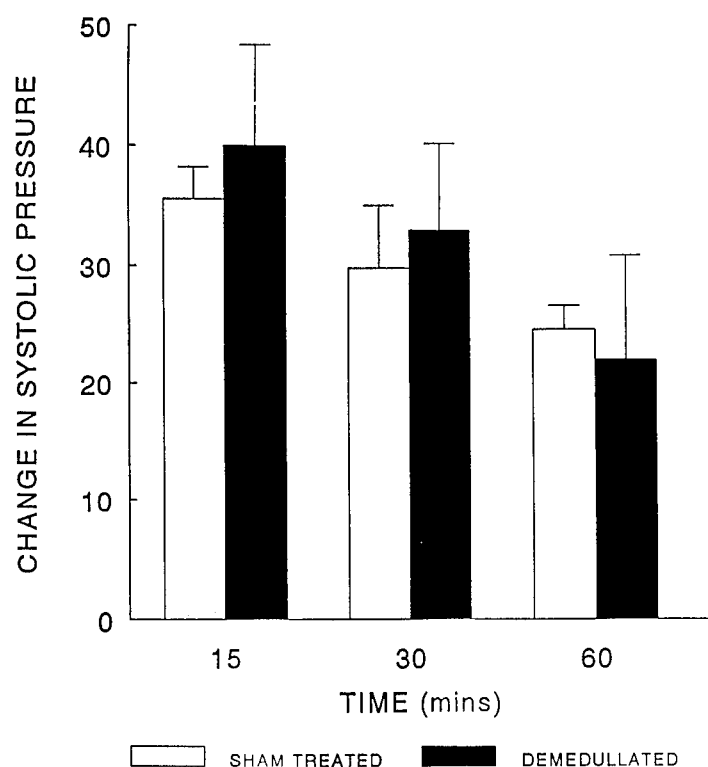
Fig. 2 The effect of DCLHb (400 mg/kg, i.v.) on the change in systolic blood pressure (mmHg) in sham treated (blank bars) and bilateral adrenal demedullated (filled bars) rats.

Clonidine (75 μg/kg, iv) produces hypotension and bradycardia 15 mins after its administration (Figs. 3 and 4). It was noted that DCLHb pretreatment antagonized the hypotensive ($F(2,12) = 11.56$, $P = 0.0067$) and bradycardic ($F(2,9) = 77.53$; $P < 0.0001$) effects of clonidine. Yohimbine (2 mg/kg, iv) pretreatment prior to the administration of DCLHb and clonidine completely blocked the clonidine induced effect on blood pressure.

Effect of DCLHb on clonidine responses in cervical sectioned rats

The central hypotensive effect of clonidine was not seen in cervical sectioned rats (Fig. 5). A low dose of clonidine (25 μg/kg, i.v.) produced some pressor effect, but there was no effect on heart rate. DCLHb (400 mg/kg, i.v.) pretreatment significantly ($F(3,17) = 20.83$; $P = 0.0007$) potentiated the pressor effect of clonidine, while heart rate was not affected. After prazosin (1 mg/kg, i.v.)

GULATI AND REBELLO
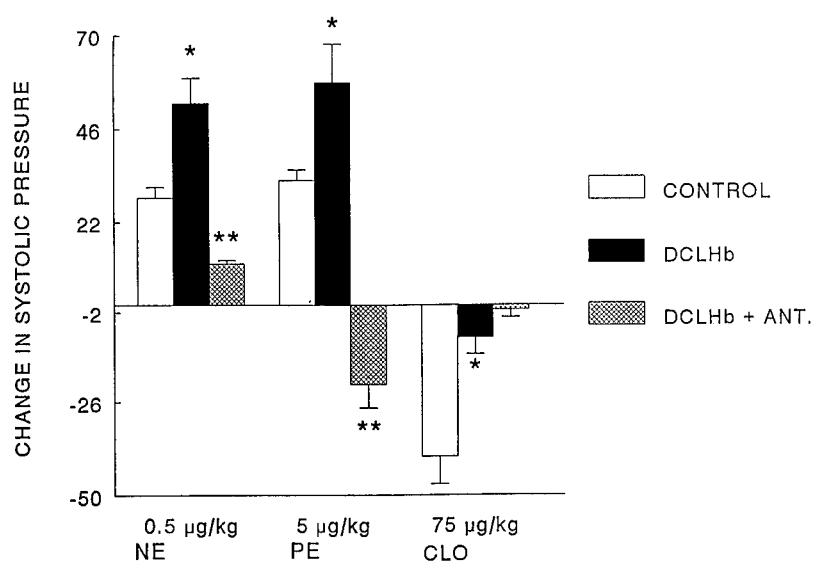
Fig. 3 The effect of DCLHb on the adrenergic agonist induced change in systolic
blood pressure (mmHg).   Blank bars indicate control responses to
norepinephrine (0.5 μg/kg, iv), phenylephrine (5 μg/kg, iv) and clonidine (75
μg/kg, iv). The effect of 15 min DCLHb (400 mg.kg, iv) pretreatment on
norepinephrine, phenylephrine and clonidine responses are shown as filled
bars.   The hatched bars indicate the effect of 15 min pretreatment of
DCLHb and phenoxybenzamine (5 mg/kg, iv) or prazosin (1 mg.kg, iv) or
yohimbine (2 mg/kg, iv) on norepinephrine, phenylephrine and clonidine
responses, respectively. *Indicates significant difference as compared to
control rats; **Indicates significant difference as compared to DCLHb
induced effects.

pretreatment, the potentiation of the pressor effect of clonidine by DCLHb was
significantly ($F_{(3,17)}$ = 20.83; P = 0.0007) blocked. However, the blockade by
yohimbine (2 mg/kg, i.v.) pretreatment was more significant ($F_{(3,17)}$ = 20.83, P
= 0.0002).

DISCUSSION

The present studies confirm earlier observations (10) that DCLHb produces
a pressor effect in rats. When given intravenously in cervical sectioned rats,
DCLHb produced an increase in blood pressure comparable to that observed in
normal rats. Since DCLHb produced its pressor effect when the influence of the
central nervous system in blood pressure regulation was removed, it is clear that
the pressor effect of DCLHb was mediated through the peripheral vascular system
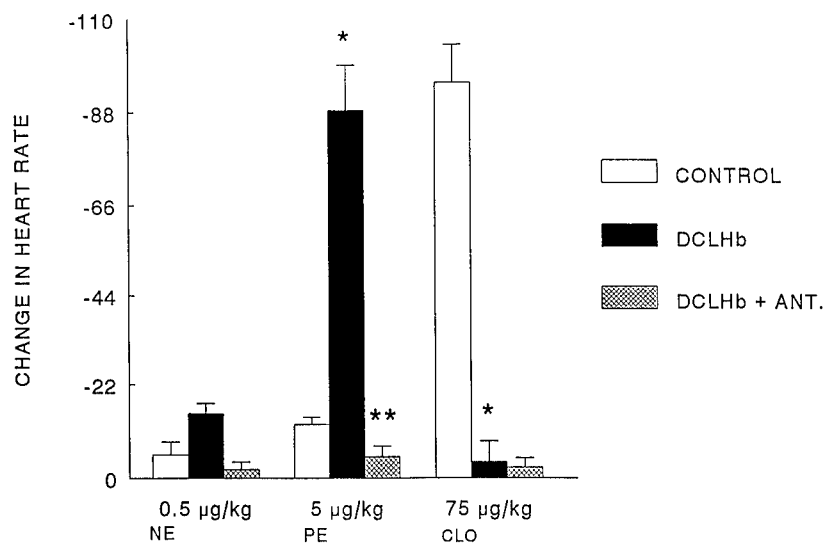
Fig. 4 The effect of DCLHb on the adrenergic agonist induced change in heart rate (beats/min). Blank bars indicate control responses to norepinephrine (0.5 μg/kg, iv), phenylephrine (5 μg/kg, iv) and clonidine (75 μg/kg, iv). The effect of 15 min DCLHb (400 mg.kg, iv) pretreatment on norepinephrine, phenylephrine and clonidine responses are shown as filled bars. The hatched bars indicate the effect of 15 min pretreatment of DCLHb and phenoxybenzamine (5 mg/kg, iv) or prazosin (1 mg.kg, iv) or yohimbine (2 mg/kg, iv) on norepinephrine, phenylephrine and clonidine responses, respectively. *Indicates significant difference as compared to control rats; **Indicates significant difference as compared to DCLHb induced effects.

rather than through the central nervous system. In order to investigate the role of the adrenal medulla in the pressor effect of DCLHb, experiments were performed in bilateral adrenal demedullated rats. DCLHb produced a similar pressor effect in bilateral adrenal demedullated rats and normal rats. It is thus clear that DCLHb does not produce a pressor response by releasing catecholamines or other pressor substances from the adrenal medulla. It is therefore possible that DCLHb alters the vascular sensitivity of α-adrenoceptors which, when stimulated, will produce vasoconstriction and an increase in blood pressure.

DCLHb pretreatment significantly potentiated the pressor response to norepinephrine. The specificity of the potentiation was confirmed by using phenoxybenzamine, a specific α-adrenoceptor antagonist (13), which completely blocked the DCLHb induced potentiation of norepinephrine responses. Studies were carried out to determine the subtypes of α-adrenoceptors sensitized by DCLHb. DCLHb significantly potentiated the pressor and heart rate effects of
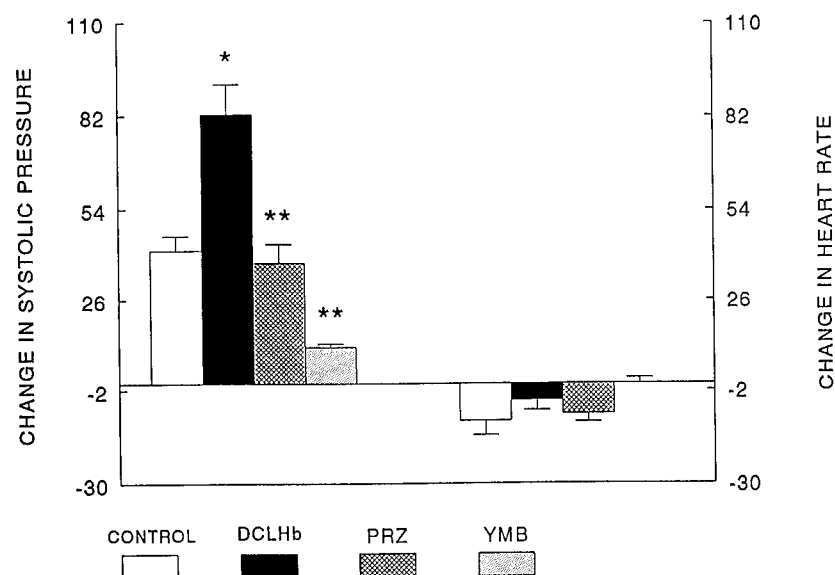
Fig. 5 The effect of DCLHb on the change in systolic blood pressure (mmHg) and heart rate (beats/min) induced by clonidine (25 μg/kg, i.v.) in cervical sectioned rats. Clonidine was given alone in sectioned rats (blank bars), 15 min after treatment with DCLHb (400 mg/kg, i.v.) (filled bars), 15 min after treatment with DCLHb (400 mg/kg, i.v.) and prazosin (1 mg/kg, i.v.) (hatched bars), and 15 min after treatment with DCLHb (400 mg/kg, i.v.) and yohimbine (2 mg/kg, i.v.) (dotted bars). *Indicates significant difference as compared to control rats; **Indicates significant difference as compared to DCLHb induced effects.

phenylephrine, a specific a_1-adrenoceptor agonist. The potentiation of a_1-adrenoceptor mediated responses by DCLHb was blocked by a specific antagonist, prazosin, acting on the a_1-adrenoceptors (14). Clonidine produces a fall in blood pressure by acting on the central a-adrenoceptors (15,16,17) and a rise in blood pressure by stimulating the peripheral vascular a-adrenoceptors (18,19). DCLHb produced a marked potentiation of the pressor response to clonidine to the extent that the central hypotensive effect of clonidine was not observed in DCLHb pretreated rats. The effect of clonidine and DCLHb plus clonidine was completely blocked by yohimbine, an a_2-adrenoceptor antagonist (13), pretreatment. Further studies were performed in cervical sectioned rats, where the central hypotensive effect of clonidine was not present. DCLHb markedly potentiated the pressor effect of clonidine in cervical sectioned rats. The potentiation of the clonidine induced pressor response by DCLHb could be attenuated by prazosin. Yohimbine completely blocked the pressor effect of clonidine in cervical sectioned rats.

Therefore, it appears that both a_1- and a_2-adrenoceptors in the peripheral vascular system are sensitized by DCLHb.

Exchange transfusion with pyridoxalated hemoglobin polyoxyethylene conjugate (PHP) did not alter the constrictor responses to angiotensin II and 5-HT (20). However, the responses to norepinephrine were significantly augmented in PHP transfused rats (20). These findings support our observations that hemoglobin increases the sensitivity of vascular a adrenoceptors. The mechanisms involved in increasing the sensitivity of vascular a adrenoceptors are not known.

It can be concluded that the pressor effect of DCLHb is not mediated through central nervous system or adrenal medullary mechanisms, but appears to be mediated at least in part through the peripheral vascular system. DCLHb potentiates the pressor responses to norepinephrine, phenylephrine and clonidine possibly due to the increased sensitivity of peripheral vascular a-adrenoceptors. The specific role of other mediators such as EDRF (NO), endothelin, etc. were not addressed in these studies.

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COAGULATION RESPONSES OF HUMAN PLASMA AFTER HEMODILUTION WITH HEMOGLOBIN SOLUTION   IN-VITRO

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ABSTRACT

To assess the potential for interference of Hb with the normal coagulation mechanism, we performed in-vitro hemodilution tests. Platelet rich plasma(PRP) was prepared from citrated blood samples of 5 normal volunteers diluted 3:1, 1:1, and 1:3 volume ratio with human stroma-free hemoglobin solution (SFH) or human albumin (HSA). Coagulation kinetics and clot strength were assessed with a thrombelastograph(TEG). Extrinsic and intrinsic coagulation factors were assessed measuring prothrombin time (PT) and activated partial thromboplastin time (aPTT) with an optical coagulation timer. Statistical significance was assessed using ANOVA and Neuman-Keuls tests at $p<0.05$. At all dilutions, SFH diluted plasma showed significantly prolonged initial rate of clot formation compared to undiluted control or HSA ($p<0.05$). However, there was no difference in formed clot strength between SFH and HSA. At high Hb concentrations Hb seems to interfere with the optical measurements of coagulation times (particularly aPTT). SFH appears to interfere with

the initial phase coagulation mechanism in human plasma
in-vitro; further study is needed to clarify the cause.
In measuring coagulation times of plasma containing Hb a
non-optical instrument should be considered.

INTRODUCTION

Clinically useful hemoglobin-based oxygen carriers (HBOC)
must not interfere with normal coagulation mechanism.
However, studies in experimental animals (1-4) and human
subjects (5,6) infused with hemoglobin (Hb) solutions have
shown widely varying responses ranging from no effect
(2,4,6) to hyper (2,3) or hypocoagulation (5). Differences
in test Hbs used (tetramer or polymer, modifiers,
modification sites, etc), degree of purity (metHb, stromal
phospholipids, endotoxin, residual red cell enzymes, etc),
test methods, and model differences could have contributed
to such diverse responses. However, the relationship
between pure Hb and the coagulation mechanism has never
been clearly established. To help understand this
fundamental Hb coagulation mechanism relationship, we
investigated the effects of human stroma-free hemoglobin
(SFH), a common raw material of most HBOCs, on human blood
coagulation dynamics in-vitro.

MATERIALS AND METHODS

Platelet rich plasma(PRP) was prepared from the citrated
blood samples of 5 normal volunteers. Platelet counts were
performed with a phase contrast microscope (Nikon, Model
TMS) using standard methods. Aliquots of PRP from the same
donor were then diluted 3:1, 1:1, and 1:3 volume ratio
with human stroma-free hemoglobin solution (SFH, 7gHb/dl)
or human albumin solution (HSA, 5g/dl). Coagulation
kinetics and clot strength were assessed using a
thrombelastograph (TEG, Hellige, Germany) as described
previously (7,8). TEG parameters r- and k-times reflect

the status of initial phase of the coagulation mechanism (factors XII, XI, IX, VIII, etc); shortened or prolonged r- and k-times would indicate hyper or hypocoagulopathy, respectively. The maximum amplitude (Ma) on TEG recording represents maximal clot strength which is related to the degree of fibrin polymerization and crosslinking (factor XIII). Classic extrinsic and intrinsic coagulation pathways were also tested by the more conventional prothrombin time (PT) and activated partial thromboplastin time(aPTT) with an optical coagulation timer (MLA 750A, MLA Inc., New York). Statistical significance was assessed using ANOVA and Neuman-Keuls tests at p<0.05.

RESULTS AND DISCUSSION

Platelet counts of all samples tested were within the normal range (200,000-300,000/mm^3). At all dilutions, SFH diluted plasma showed a significantly prolonged r-time compared to undiluted control or HSA (p<0.05). However, although dilutional effect was clearly notable, there was no statistical difference in the formed clot strength (Ma) between the SFH and HSA groups (P>0.05). In addition, no statistical differences in PTs and aPTTs were found between the two groups. Of note, at higher Hb concentrations (1:1 and 1:3) the coagulation timer did not detect clot formation consistently indicating a possible Hb interference with the optical measurements of coagulation times. This was particularly a problem for aPTT measurements making it unmeasurable at the highest Hb concentration tested.

The hypocoagulative effect of SFH observed in this study is somewhat unexpected as we have previously observed a moderate hypercoagulative tendency with rat blood hemodiluted with human SFH (8). One possible cause of the difference is that rats have much higher platelet counts than humans (over 1 million versus 250,000/mm^3). On the

TABLE-I. Coagulation Responses of Human Plasma after Dilution with Human SFH In-vitro

| | Undiluted PRP | Diluted (PRP:SFH or HSA) | | | | | |
| | | 3:1 | | 1:1 | | 1:3 | |
	PRP	SFH	HSA	SFH	HSA	SFH	HSA
r(min)	12.7 ±1.3	17.5* ±2.3	11.8 ±.8	27.1* ±3.0	10.8 ±1.2	35.4* ±5.7	9.3 ±3.0
Ma(mm)	48.7 ±1.8	38.1 ±4.1	41.6 ±4.2	27.3 ±8.4	33.1 ±6.0	9.8 ±7.7	22.0 ±4.9
PT(sec)	12.7 ±.24	13.5 ±.57	13.4 ±.14	ND	ND	18.0 ±.0	18.0 ±.21
aPTT(sec)	26.2 ±4.2	23.2 ±.21	23.0 ±.35	ND	ND	UM	>100

* Significantly different from HSA (P<0.05)
ND: not determined, UM: unmeasurable

other hand, human Hb may interact differently with the rat
platelets. Further studies are required to clarify these
questions. The observation that SFH interferes with a
methodology for assessing coagulation could lead to over
or under interpretation of the data as presented in the
literature. That is, some of the variation in coagulation
in response to SFH infusion may be a methodologic
artifact. Moreover, this study represents one of the few
in which human SFH is tested in-vitro with human blood.
In-vitro tests indicate that stroma-free Hb seems to
interfere with the initial phase coagulation mechanism;
further study is needed to clarify the cause. The presence
of Hb in the plasma in-vitro also seems to interfere with
coagulation time measurements when using an optical
coagulation timer. An alternative method should be
considered when measuring samples that contain plasma Hb.

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IRON-58 AND NEUTRON ACTIVATION ANALYSIS: A NON-RADIOACTIVE METHOD FOR TRACING HEMOGLOBIN IRON

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ABSTRACT

To develop a non-radioisotopic altnerative to radioactive Fe-59 as a hemoglobin iron tracer, we evaluated Fe-58 and neutron activation analysis (NAA). Upon irradiation with thermal neutrons, stable isotope Fe-58 is converted to radioactive Fe-59 through (n,gamma) neutron reaction; its radioactivity measured by a gamma counter is proportional to the original Fe-58 present. Organ samples from rats infused with Hb or saline were analyzed along with a 10 mg Hb as a standard (105 nanograms of Fe-58). The measured values of Fe-58 in tissues were compared with those derived from the literature tissue iron contents. The amount of Fe-58 in normal tissue is extremely low yet detectable by NAA demonstrating high sensitivity. Measured values of Fe-58 from tissues were generally correlated well with literature derived values (e.g., brain: 0.19 and 0.15 ng/mg tissue; kidney: 0.6 and 0.7 ng/mg tissue, respectively). The results indicate that Fe-58 can be used as a non-radioactive tracer for absorption, distribution, metabolism, and distribution studies of hemoglobin-based oxygen carriers.

INTRODUCTION

Because hemoglobin (Hb) contains a relatively large amount
of iron (3.5mg Fe/ gHb), there is a potential risk for
iron related toxicities (cell/tissue damage by reactive
oxygen metabolites, vasoconstriction by interacting with
endothelium derived relaxation factor, potentiation of
bacterial infection, etc) in the use of hemoglobin-based
oxygen carriers (HBOC). Absorption, distribution,
metabolism, and excretion (ADME) of hemoglobin iron must
be investigated as a part of HBOC safety evaluation [1].
Conventional methods of studying Hb iron metabolism
involve the use of radioactive iron, Fe-59, as a tracer.
In experimental animals, Fe-59 labeled Hb is generally
produced by "in-vivo labeling" as described by Beguine,
et al. (2); radioactive Fe-59 is injected intra-
peritoneally into an anemic animal and the label is
incorporated into new Hb "in-vivo" through erythropoiesis.
Blood samples are then collected from the animal and
processed to obtain Fe-59 labeled Hb. As iron absorption
and incorporation rate is relatively low in normal
animals, the donor animal usually has an induced anemia
from repetitive hemorrhage. "In-vivo" production of Fe-59
labeled human Hb is not practical for many reasons.
Further, radioactive Hb will have to be infused into an
experimental subject to assess metabolic data; this
requires radiation exposure. To develop a non-radioactive
method for studying the ADME of HBOC iron, we have
investigated use of a stable iron isotope, Fe-58, as an
exogenous hemoglobin iron tracer and "post hoc" analysis
of samples by neutron activation.

MATERIALS AND METHODS

Principles of Neutron Activation Analysis of Iron-58

When irradiated with a neutron source, the common Fe-56
is converted to short lived radioactive Mn-56 through

(n, p) neutron reaction while the stable isotope Fe-58 converted to long lived radioactive Fe-59 through (n,gamma) neutron reaction (3).

```
        (n, p)                        gamma-ray
Fe-56 -----------> Mn-56 (T1/2=2.6 hrs.) ---------> Fe-56

        (n, gamma)                    beta, gamma-ray
Fe-58 -----------> Fe-59 (T1/2=45 days) ----------> Co-59
```

The radioactivity of the reaction product, Fe-59, is proportional to the original Fe-58 present and can be measured using a gamma-counter. The stable iron isotope Fe-58 occurs naturally only in 0.3% of iron making it ideal as a tracer for HBOC iron. Enriched Fe-58 labeled human Hb can be safely produced in human subjects allowing Hb metabolic studies be performed without radiation exposure to the subject.

Experimental Protocol

Male Sprague-Dawley rats weighing 200-250 grams were used. The rats were anesthetized with sodium pentobarbital (70-80mg/Kg, IP) and one third of estimated blood volume (2% of body weight) was withdrawn from the right jugular vein catheter and immediately replaced with shed blood volumes of stroma-free hemoglobin (SFH) or normal saline solution (NSS). Two hours after replacement, the animals were exsanguinated and perfused with 100ml heparinized saline to wash out red cells through the ascending aorta. Organ samples (brain, heart, lungs, liver, spleen, and kidneys) were then harvested. After lyophilization the organ samples were ground to powder and carefully weighed into the polyethylene activation vials. The samples along with 10mg of dried Hb (105ngm of Fe-58) as a standard were irradiated with a thermal neutron source for 4 days at a neutron flux level of $4x10^{12}$ neutrons/cm^2/second. Radioactivity of Fe-59 was measured by a gamma spectroscope equipped with a Ge-Li detector. The measured

KIM, YU, AND GREENBURG

TABLE 1. Fe-58 Measured by Neutron Activation Analysis
(NAA)

Sample	Counts/hr/mg sample (1.099MeV peak)		Measured values[1]	Reference values[2,3]
			(ngm Fe-58/mg sample)	
Blank vial	0.098±0.008		0	-
Hb(standard)	222.2±38.3		10.5[4]	N/A
	SFH Rats	NSS Rats	SFH NSS	
Brain	4.1±1.1	3.8±0.1	0.19 0.18	0.15
Heart	11.3±4.7	11.0±0.6	NC NC	N/A
Lung	13.0±2.7	8.7±3.5	NC NC	N/A
Liver	10.1±2.4	10.8±3.4	0.5 0.53	0.36
Spleen	50.6±13.8	67.9±38.8	NC NC	N/A
Kidney	12.6±1.6	7.7±1.7	0.6 0.36	0.71

Values are mean±1 S.D. (N=2 each).
1. Calculated Fe-58 = (Counts/mg)/(Hb counts/mg) x
   Hb Fe-58 (ngm.)
2. Reference values of Fe-58:
   Fe-58 = (Literature value of Fe/mg tissue) x 0.003
3. Source: see reference 4, 5.
4. Hb Fe-58(ngm.)= 3.5ugFe/mgHb x 0.003 x1000

SFH: stroma-free Hb, NSS: normal saline solution,
N/A : not available, NC: not calculated.

values of Fe-58 from tissues were compared with those calculated from literature tissue iron contents (4,5).

RESULTS AND DISCUSSION

The gamma spectrum showed two energy peaks for Fe-59, 1.099 and 1.291 MeV. Sodium-24 also has a peak at around 1.3 MeV energy level and high sodium contents in the tissues presented an interference with detection of Fe-59. However, after a two-week "cooling" period, sodium-24 (T1/2= 15 hours) and other short-lived nuclides had decayed sufficiently to allow reliable measurements of Fe-59 (T1/2=45 days) radioactivity. Since the 1.099 MeV peak produced higher counts it was used to calculate Fe-58 content. The amount of Fe-58 in normal tissue is extremely low yet detectable by NAA demonstrating high sensitivity. As shown in Table-1, measured values of Fe-58 from various tissues generally correlated well with tissue iron levels reported in the literature (4,5). Of note, Fe-58 levels in the kidneys of SFH infused rats were substantially higher than those of the saline rats, an expected finding because SFH is cleared rapidly through the kidneys. The results clearly demonstrate that Fe-58 can be used as a non-radioactive tracer for ADME studies of HBOC iron.

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DENATURED HEMOGLOBIN INCREASES HUMAN BLOOD MONONUCLEAR CELL PROCOAGULANT EFFECT

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ABSTRACT

Purpose: To study the effects of the common contaminants of hemoglobin solutions, red cell stroma, bacterial endotoxin, and denatured hemoglobin on the causes of the thrombotic lesions which have been reported in animal experiments after hemoglobin administration.

Protocol: Human blood mononuclear cells were isolated on Ficoll-Hypaque gradients and incubated with hemoglobin from the LAIR production facility, red cell stroma, bacterial endotoxin (E. coli, Wittaker Bioproducts), and hemoglobin denatured by boiling. Incubations were performed separately and in combinations. Mononuclear cells were then lysed and assayed for procoagulant activity in a recalcification time assay.

Results: Only bacterial endotoxin and hemoglobin denatured by boiling increased the procoagulant activity of human blood mononuclear cells. Denatured hemoglobin mixed one part in eight with undenatured hemoglobin increased mononuclear cell procoagulant activity by more than ten-fold that of the undenatured hemoglobin control.

Conclusions: The study suggests that denatured but not undenatured hemoglobin causes increased blood procoagulant activity which is thought to be a marker of macrophage activation. These findings suggest a possible mechanism of toxicity of cell-free hemoglobins and the need for sensitive measures of hemoglobin denaturation.

INTRODUCTION

The toxicity of cell-free hemoglobin (Hb) has been difficult to define because mechanisms of toxic action are unexplained and because trace contaminants are present in all Hb preparations. Understanding the interaction of Hb with the immune system has been a particular problem [1]. Fever and inflammatory lesions have been seen variably after administration of a variety of Hb products and in a number of testing situations [2]. The inconsistency of observed inflammatory toxicity has led to the frequent assumption that unrecognized contamination of the Hb products was responsible for the toxicity.

The common contaminants of cell-free Hb are red blood cell stroma, breakdown products of contaminating bacteria, and breakdown products of Hb itself. Cell wall phospholipids, bacterial endotoxin, and heme cause known toxicity.

We have attempted to determine if cell-free Hb separate from its contaminants increases human mononuclear cell procoagulant activity. We have tested for this activity after exposing cells to sterile, HPLC purified human Hb and combinations of Hb and freshly isolated stroma, isolated bacterial endotoxin, and denatured Hb. Denatured Hb and endotoxin cause the response, but native Hb or stroma do not.

MATERIALS AND METHODS

HbA_0 was isolated from stroma-free hemolysate (SFH) by high performance liquid chromatography (HPLC). The SFH was made by lysing outdated banked RBCs with hypotonic phosphate buffer and successive cross-flow filtering at 0.65 μm and .011 μm (300KD cut-off). HPLC was performed on a 10 x 90 cm Mono-Q column with saline gradients on a Waters Kiloprep at 4°C. The HbA_0 was formulated as a 6 g/dl solution in Ringer's acetate. Endotoxin was less than 0.06 EU/ml and organic phosphate was less than 1 μg/ml.

Human mononuclear cells were isolated from heparinized venous blood first as buffy coat and then as enriched buffy coat by centrifugation and finally as mononuclear cell suspension on Ficoll-histopaque density gradients. Cells were washed and maintained in HEPES-buffered saline. Cell isolation was confirmed by differential counting, α-naphthyl acetate esterase staining, and trypan blue staining.

RBC stroma were prepared from the precipitate of trichloromethane extracted, triply frozen, fresh whole blood. The precipitate was washed and suspended in HEPES saline.

Bacterial endotoxin from *E. coli* 0127:B8 was purchased from Sigma Chemical Co., St. Louis, MO.

Denatured hemoglobin was made by removing 1/8 part (0.5 ml of 4 ml) of the HbA_0 solution, heating it to 100°C for 10 minutes, and adding it back to the undenatured fraction.

Isolated cell fractions were divided and mixed with buffer or contaminant, and then divided again, with half of the material frozen immediately and the other half incubated in parallel with its control for 20 hours at 37°C. This pattern of parallel incubation with preincubation controls

was repeated for cells from each donor (n=3) with HbA_0 alone and with HbA_0 and added endotoxin, stroma, and denatured Hb.

Measurement of procoagulant activity was performed with freeze-thawed, n-octyl-α-D-glucopyranoside solubilized, and sonicated cell suspensions in a one-stage clotting time measured in a fibrometer (Dataclot 2, Helena Labs, Beaumont, TX). Activity was measured as units of thromboplastic activity against a rabbit brain standard. Procoagulant activity ratios were calculated from clotting time ratios normalized for protein content as (Post-incubation activity with Hb & contaminant/Post-incubation activity with vehicle alone)/(Pre-incubation activity with Hb & contaminant/Pre-incubation activity with vehicle alone).

RESULTS

Preliminary experiments showed a marked variability in mononuclear cell procoagulant activity amoung freshly isolated cells of different donors. The increase in activity with exposure to Hb or a contaminant was thus expressed as an activity ratio with the unincubated sample as the control. The increase in procoagulant activity with increasing endotoxin exposure was also dose dependent, but varied among donor's cells.

In this system clotting time did not increase with incubation for 20 hr with HbA_0. Data from a single patient are shown in FIGURE 1.

Procoagulant activity increased with incubation with endotoxin and denatured Hb. Data from a single patient are shown in FIGURE 2.

DISCUSSION

Mononuclear cell procoagulant activity is believed to be tissue factor protein which is normally present in small amounts inside cells and is synthesized and externalized in response to activating stimuli. The

PROCOAGULANT ACTIVITY OF MONOCYTES
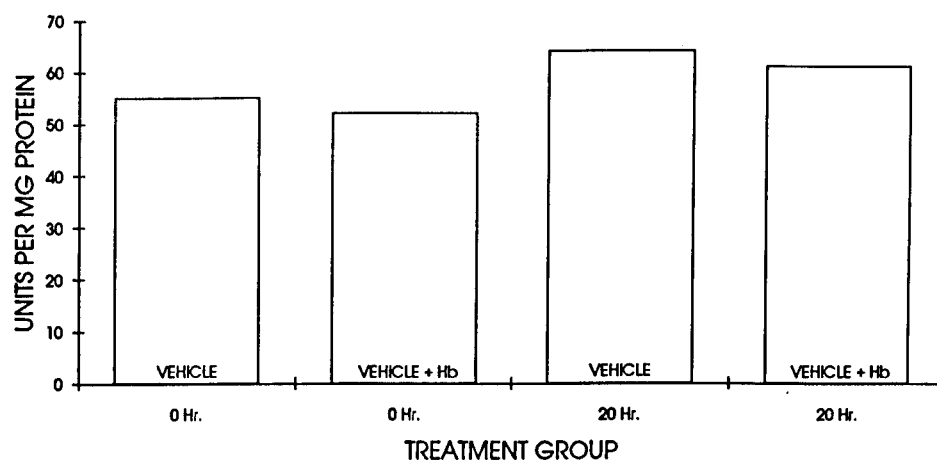
FIGURE 1. Procoagulant activity of human monocytes from one donor exposed to HbA_0 and saline vehicle for 0 and 20 hours. The minimal increase with incubation appears to be nonspecific because a similar increase occurred with exposure to the saline control. The activity ratio calculated from these measures is about 1.2.

PROCOAGULANT ACTIVITY RATIO
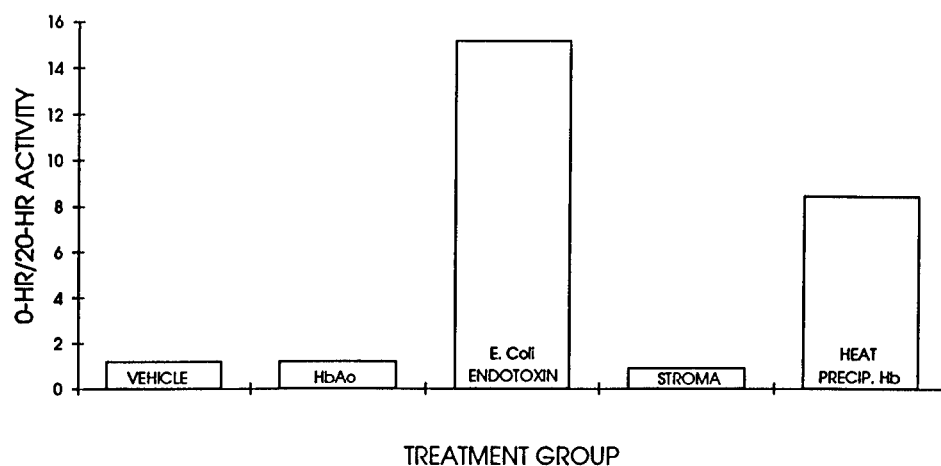
FIGURE 2. Procoagulant activity ratio for the cells of one donor after the cells were exposed for twenty hours to vehicle, HbA_0, and Hb and contaminants as listed. Endotoxin and heat denatured Hb consistently caused elevations about 10 times baseline values.

KIM, VILLA, AND HESS

experimental design was developed to allow control of the variability in mononuclear cell procoagulant activity expression between white cell donors and to differentiate increases in activity associated with handling from those associated with exposure to potentially stimulating agents.

We found that incubation with human Hb that had been purified by HPLC and that contained concentrations of bacterial endotoxin below 0.06 EU/ml did not increase the human mononuclear cell procoagulant effect. This result is at variance with previous work from our lab [3].

Bacterial endotoxin and denatured Hb strongly and independently increased the human mononuclear cell procoagulant effect. Endotoxin is known to activate this response. Denatured hemoglobin separate from hemoglobin has not previously been reported to cause this reaction. Whether a specific reaction to the denatured Hb or a nonspecific reaction to denatured protein is responsible is unknown.

RBC stroma did not independently increase the procoagulant effect. Stroma has been reported as toxic in the past [4].

We conclude that Hb denaturation and therefore storage stability may be critical issues in Hb safety. While good manufacturing practice can insure low levels of contamination with phospholipids and bacterial endotoxin, proper post-manufacture handling is necessary to prevent the development of hemoglobin breakdown products. More sensitive measures of Hb denaturation should be developed.

ACKNOWLEDGEMENTS

Human Subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Reg 50-25 on the use of volunteers in research.

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MIXTURES OF ERYTHROCYTES AND ACELLULAR FLUIDS: AN IN VITRO EVALUATION

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ABSTRACT

To evaluate the biophysical properties of two acellular resuscitative fluids, experiments were performed using mixtures of these with suspensions of red blood cells.

Two acellular resuscitative fluids were synthesized using a diacid to tetramerically stabilized hemoglobin and then complexing it with either a hydroxyethyl starch or a Tetronic Polyol. The new polymers were characterized with respect to the molecular weight, second virial coefficient and intrinsic viscosity. These fluids were then combined with red cells and the following measurements were made: non-Newtonian flow properties, malonamide induced hemolytic kinetics, sedimentation rates and oxygen transport.

The in vitro evaluation indicates that these compounds are effective hemodiluents, offer protection to the red cell membrane and do not cause erythrocyte aggregation. The oxygen transport was satisfactory.

INTRODUCTION

It is possible to synthesize a suitable acellular resuscitative fluid from a modified hydroxyethyl starch

(HES) and a tetramerically stabilized hemoglobin (Hgb).
These initial studies have been extended to include a
Tetronic Polyol complexed with hemoglobins treated
with a variety of low molecular weight diacids. The
polymers provide the needed oncotic and rheological
properties while the stabilized hemoglobins transport
the required oxygen. It appears that these polymer-
hemoglobin complexes could serve as useful blood sub-
stitutes.

MATERIALS AND METHODS

Hemoglobin. The hemoglobin was prepared from out-
dated human blood obtained from local hospital blood
banks. The methods of separation and purification em-
ployed the Boycott Effect as previously reported [1].
Polymer Modification. The HES polymers were converted
to mono-, di- and tri-aldehyde moieties by standard-
ized methods [2,3]. To form a Tetronic Polyol Tetra-
aldehyde, the technique of Pfitzner and Moffatt [4,5]
proved to be satisfactory.
Hemoglobin-Polymer Complexes. The HES-Hgb polymers
were synthesized as previously reported [6] and the
Tetronic Polyol-Hgb compound were similar to those
prepared in an earlier publication [1].
Plasma Retention. In order to follow the retention
of the polymer complexes in the plasma, exchange-
transfusion experiments were performed essentially
following the techniques suggested by Tabata and Chang
[1]. The plasma was separated from the red cells by
centrifugation. Cellulose acetate plate electrophore-
sis patterns were used to follow the metabolic kinetics.

RESULTS

Initial in vivo exchange-transfusion experiments
in rats at a 90% replacement level using a 6% Hgb poly-
mer solution indicated a plasma retention half-life of
approximately 20 hours. However after 72 hours there
remained about 14% Hgb in the plasma. In order to bet-
ter evaluate these retention data, a cellulose acetate

electrophoresis pattern was determined at each time
point. These plates were subjected to densiometric
measurements using HES-Hgb-Cyclo, Hgb-Cyclo and Hgb as
standard references. Based upon an initial 6% Hgb con-
centration, the data could be graphically represented
as shown in Fig. 1. The following kinetic scheme ap-
pears to represent the data:

$$\text{HES-Hgb-Cyclo} \xrightarrow{k_1} \text{HES + Hgb-Cyclo} \xrightarrow{k_2} \text{Hgb} \xrightarrow{k_3} \text{out of plasma.}$$
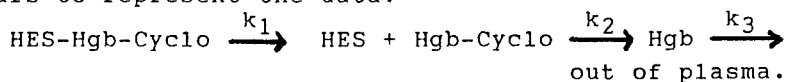
     This mechanism of consecutive reactions is similar
to a previous treatment given by Cerny [7]. The dif-
ferential equations are:

$$\frac{d\,(Polymer)}{dt} = -k_1\,(Polymer)$$

$$\frac{d\,(Hgb\text{-}Cyclo)}{dt} = k_1\,(Polymer) - k_2\,(Hgb\text{-}Cyclo)$$

$$\frac{d\,(Hgb)}{dt} = k_2\,(Hgb\text{-}Cyclo) - k_3\,(Hgb)$$

     These equations can be integrated using the
proper boundary conditions to yield:

$$(Polymer) = 6\,e^{-k_1 t}$$

$$(Hgb\text{-}Cyclo) = \frac{6\,k_1}{(k_2 - k_1)}\,(e^{-k_1 t} - e^{-k_2 t})$$

$$(Hgb) = 6\,k_1 k_2 \left[\frac{1}{(k_2 - k_1)} \left[\frac{e^{-k_2 t}}{(k_2 - k_3)} - \frac{e^{-k_1 t}}{(k_1 - k_3)} \right] + \frac{e^{-k_3 t}}{(k_2 - k_3)(k_1 - k_3)} \right]$$

A preliminary treatment of the data suggests that $k_1 =$
0.14 hr^{-1}; k_2 = 0.0829 hr^{-1} and k_3 = 0.204 hr^{-1} for a
polymer solution containing 6% Hgb. These data are
shown in Fig. 1. The agreement is satisfactory. What
should be noted are the kinetic curves for (Hgb-Cyclo)
and (Hgb). The maximum values for these components
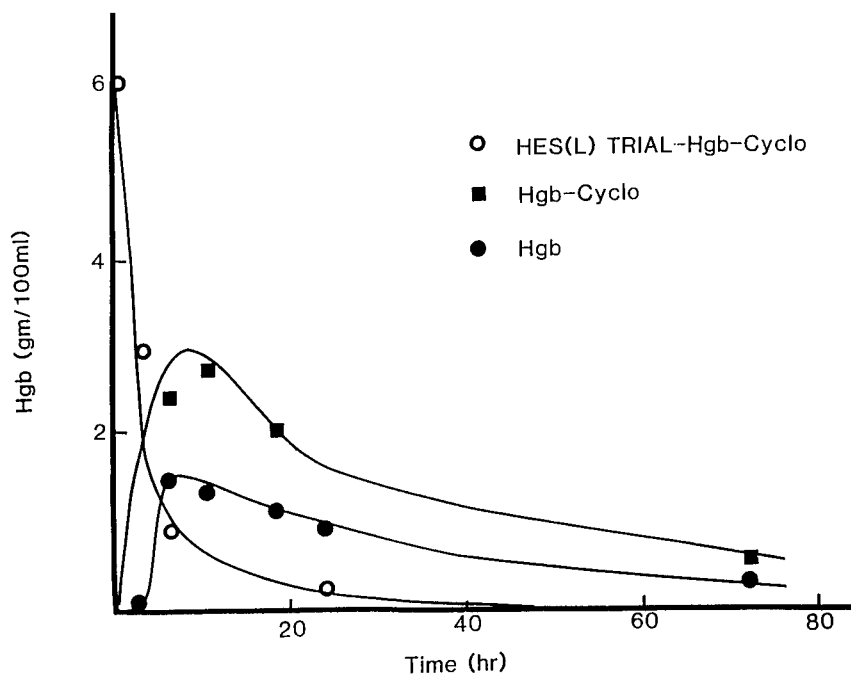
FIGURE 1.   Plasma Retention as a function of time

show that there is an increased retention time of the
Hgb in the circulation because of the sequential meta-
bolic mechanism of the polymer formed between the HES
and the stabilized Hgb.   These preliminary data suggest
the Hgb concentration becomes maximum at about 16 hours
after the exchange-transfusion is completed.   After
this time, the Hgb is removed from the plasma exponen-
tially.   Therefore it appears that if the Hgb concen-
tration in the original polymer can be increased sub-
stantially, then the effectiveness of the oxygen trans-
port from the Hgb remaining in the plasma can be con-
trolled.   The parameters available to attain this goal
are as follows: The degree of aldehyde content, the
molecular weight and molecular substitution of the HES

as well as the effectiveness of the Hgb tetrameric sta-
bilization.

The shear dependent non-Newtonian viscosity was
measured in a Wells-Brookfield cone-plate viscometer at
25C. It is important to evaluate the yield stress, τ_y,
which is the minimum stress value in order for the sys-
tem to start to flow. The Casson equation is used [8]

$$\tau^{\frac{1}{2}} = \tau_y^{\frac{1}{2}} + (b\,\dot{\gamma})^{\frac{1}{2}}$$

In this equation, τ is the shear stress, $\dot{\gamma}$ is the rate
of shear and b is a parameter related to the Newtonian
flow behavior of the system. The numerical values of
the parameters are given below:

TABLE I

SAMPLE		$\tau_y(dyne/cm^2)$	b(poise)
Whole blood	51% Hct	0.436	0.0475
Mixture	38% Hct	0.041	0.0449
Mixture	31% Hct	0.026	0.0428

It is significant to note that the decrease in the mag-
nitude of τ_y not only shows the hemodilution proper-
ties of 908 Tet Hgb Glut but more importantly, it does
not cause aggregation of the red cells in the presence
of plasma which could cause blood flow to stop in the
very small vessels. The small changes in b indicate
the viscosity of a 6% 908 Tet Hgb Glut solution is very
similar to the viscosity of the plasma.

DISCUSSION

It is possible to synthesize acellular resuscita-
tive compounds from the aldehyde moieties of both hy-
hroxyethyl starch and Tetronic Polyols which can be
complexed with hemoglobins stabilized with simple or-
ganic diacids.

This series of compounds can be freeze-dried and
stored at room temperature for long periods of time.
Some of the biophysical advantages include:
1) Long retention times, up to 72 hours;
2) Effective hemodilution properties;
3) Offering protection to the erythrocyte mem-
   brane and
4) Satisfactory delivery of oxygen to the tissues.

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APPLICATION OF AQUEOUS TWO-PHASE SYSTEM IN SEPARATION/PURIFICATION OF STROMA FREE HEMOGLOBIN FROM ANIMAL BLOOD

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ABSTRACT

Aqueous two-phase system (ATPS) is suitable for treatment of biological materials. It has been traditionally employed in the separation of product from fermentation broth, and in the extraction of large-molecule proteins from colloid solution. The separation method based on ATPS features simple, rapid and continuous operation with low equipment cost. A PEG/Salt aqueous two-phase system is studied, and developed for hemoglobin (Hb) separation from animal bloods.

Stroma free hemoglobin (SFHb) was extracted from 200ml packed red blood cells by the two-stage aqueous two-phase system and subsequently was desalted and dialyzed by ultrafiltration. It took only 2.5hr to complete overall process to purify SFHb. The purity of SFHb was analyzed by HPLC and IEF (silver staining). Large partition coefficients of Hb have been observed with proper selection of PEG molecular weight, added concentrations of PEG and salt in each phase. The SFHb of very high purity which is free of other proteins (<0.1% impurity) and phospholipids (<2% impurities) may be obtained from human packed RBCs.

A two-stage extraction design using ATPS separation concept is very simple and useful for purification of SFHb. The method may be further developed to offer potent technology for hemoglobin separation from animal blood and future production industry of blood substitutes.

INTRODUCTION

Hemoglobin (Hb) existing in animal blood is known to play the key role in gas exchange and physiological respiration. Recently hemoglobin has been a potential candidate for the raw materials of blood substitutes. The highly pure hemoglobin, which is also called stroma-free hemoglobin (SFHb), is purified mostly from human or animal's blood.

The current methods for separation and purification of Hb from whole blood include centrifugation [1], crystallization [2], adsorption [3], chromatography [4,5], ultrafiltration [6] and precipitation [7], etc.; so far they have a significant disadvantage that a high speed centrifugation step (15,000g) is essentially employed to remove cell membranes and to harvest the intracellular hemoglobin. The high speed centrifugation has been often used for lab-scale batch separation in biochemical lab, but the application for large-scale process is not appropriate and uneconomic because of its characteristics of expensive maintenance, low capacity and long operation time.

An extraction method based on aqueous two-phase system is considered for use in separation of hemoglobin from whole blood. This method features simple , rapid and continuous operation with low equipment cost and ease of scale-up [8]. There are many factors which can be manipulated to fulfill the desired partition of the target protein in the extraction system [9,10]. In addition, the system possesses an excellent ability for purification of intracellular proteins from cell membrane by a simple extraction step with low speed centrifugation (<5,000g) [11].

In this study, the effects of phosphate solution pH and PEG molecular weight on the partition of hemoglobin in PEG/phosphate aqueous two-phase system have been investigated. Based on the fundamental partition observation , a two-stage extraction process was designed for separation and purification of hemoglobin from whole blood.

MATERIALS AND METHODS

Preparation of packed red blood cell

Outdated human blood was obtained from Hsin-Chu Hospital Blood Bank. The red blood cells (RBC), isolated from whole blood by centrifugation (Hitachi centrifuge ,himac CR20B2) at 5,000 rpm for 15 mins, was washed three times with 2 vol. of 0.9% saline . For 15 ml partition, RBC were hemolyzed by addition of 3 vol. of D.I. water and were separated from larger slips of cell debris by centrifugation at 15,000 g for 60 mins.

Preparation of phase system

Polyethylene Glycol (PEG), Phosphate salt and other reagents were obtained from Merck. Stock solutions of 50% (w/w) PEG with molecule weight ranging from 600 to

6,000 and of 40%(w/w) phosphate solution were used and stored in the cold. Phase system was prepared from stock solution of PEG and phosphate solution was attained by changing the ratio of NaH_2PO_4 to K_2HPO_4 . However, when solution pH was greater than 9, additional titration with NaOH was required.

Hemoglobin was finally added to the phase system to form extraction system and separated phases by centrifugation at 1,000 rpm for 10 mins after gently shaking. Hemoglobin was reacted with Drabkin's reagent (Sigma) and determined photometrically by absorbance at 540nm.

The partition coefficient of hemoglobin in each phase system was estimated by the concentrations of hemoglobin in separate phases, and partition coefficient (K) is defined by

$$K = \frac{[Hb]_{top}}{[Hb]_{bottom}}$$

Electrophoresis assay

The analytical assay followed the method of Isoelectric focusing Pharmacia Phastsystem, PastGel IEF 3-9 with Protein Kit pI 3-9. A sample with high concentration of 10 mg/ml hemoglobin was introduced to increase the sensitivity of impurity and followed by Phastsystem manual which was modified by Feola [12].

HPLC assay

A sample for analysis of phospholipids was prepared from 10 g/dl hemoglobin solution by Folch's procedure [13]. The chromatography of phospholipids was carried out by HPLC equipment supplied by Beckman , with 250mm × 46mm Lichrosorb DIOL (5μ m) column and 60mm ×46mm Si-60 (5μm) guard column from Merck. The oven temperature was kept at 50°C and the detection wave-length was 201nm. The mobile phase included solvent A: Acetonitrile and solvent B: 80% Acetonitrile/20% , 5mM phosphate solution , pH 6.3.

RESULTS

Partition data of Hb in PEG/phosphate system are shown by Fig.1. Hb prefers to partition into the top phase with increasing phosphate solution pH. The observations suggest that the negatively charged groups on the surface of hemoglobin favor to partition toward the PEG-rich phase. It was noticed that only if the pH of phosphate solution was adjusted two units higher than pI of hemoglobin, that is pH above 9, hemoglobin could concentrate in the top phase. On the other hand, the influence of lower PEG molecular
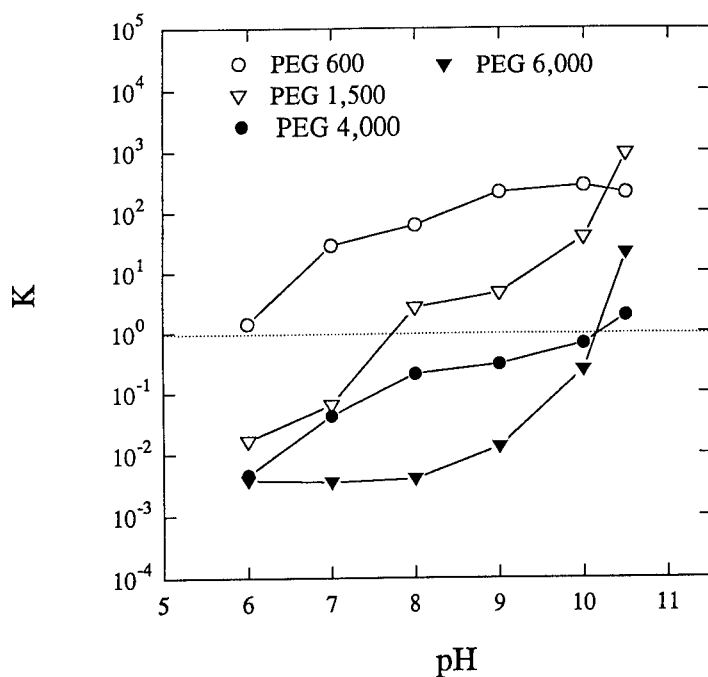
FIGURE 1   The effect of pH of phosphate phase on the partition of hemoglobin in
PEG/Phosphate system with different MW of PEG . The compositions of
different MW of PEG systems were described below: 14.% PEG 600, 14.5%
Phosphate; 12.5% PEG 1,500, 12.5% Phosphate; 9.5% PEG 4,000, 12%
Phosphate; 9.5% PEG 6,000, 10% Phosphate.

weight enhanced hemoglobin partition into the top phase. However , the large molecular
weight of PEG (>1,500) affected slightly the change of partition of hemoglobin with
varying phosphate solution pH.

Aqueous two-phase system has a characteristic that cell debris (e.g. cell membrane,
DNA and RNA) would partition strongly toward the bottom phase and the interface.
Based on the characteristic, a two-stage extraction process was designed to separate cell
membranes (phospholipids) by adjusting pH of phosphate solution and subsequently
purify hemoglobin from other impurity (proteins). The experimental procedure of the
two-stage extraction is illustrated in Fig.2. The homogenizer was adapted to lyse red
blood cells prior to the first extraction in this research, but osmotic lysis was another
available method. The top phase of the first extraction was transferred directly to form the

Packed RBCs

```
Adding D.I. water to lysis RBCs
and homogenizing for 5 min
```

```
18.9% PEG 1,500 , 9% Phosphate , pH 10
, 6 g/dl Hemoglobin
Separation by centrifugation at 5,000g for 20min
```

Bottom Phase   **Top Phase**

Discard

```
15.5% PEG 1,500 , 11% Phosphate , pH 7
Separation by centrifugation at 5,000g for 20 min
```

Bottom Phase   **Top Phase**

```
Passing through UF unit with MWCO 10,000
filter and replenished by kidney dialysis solution
```
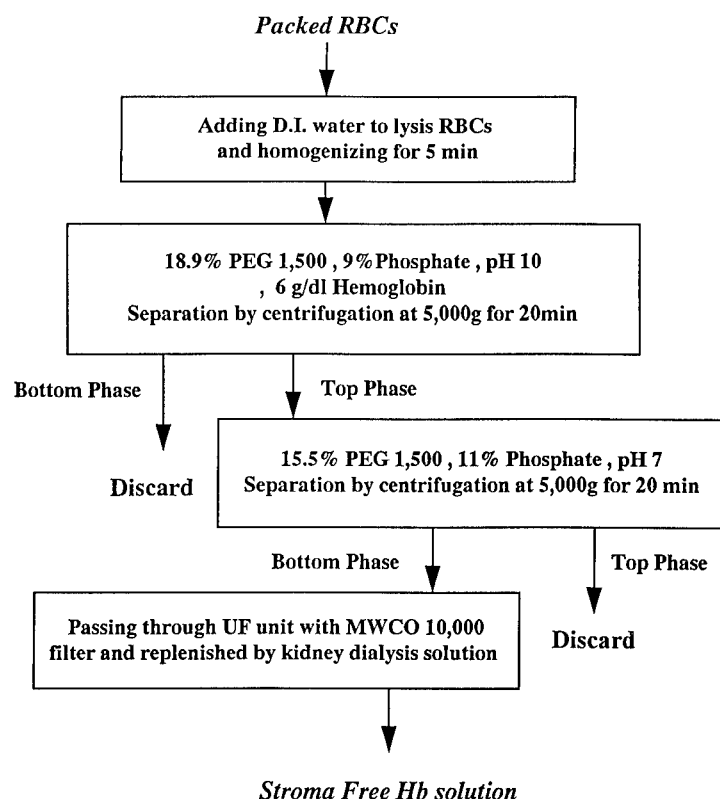**Discard**

Stroma Free Hb solution

FIGURE 2   The flow chart of the two-stage extraction.

phase system of the second extraction. It took 20 mins to perform centrifugation and 20 mins to work on the preparation for each extraction. After the two-stage extraction, ultrafiltration unit with MWCO (molecular weight cut off) of 10,000 dalton was employed to desalt and dialyze with kidney dialysis solution.

The highly pure hemoglobin solution was then obtained by this method. It took total 2.5 hours to deal with 200ml packed red blood cell. The IEF pattern is shown in Fig.3 and the chromatograms of HPLC is shown in Fig.4. The results demonstrated that most of impurity were removed. However, carbonic anhydrase (pI=6.5) was present in the final hemoglobin solution . This protein , was referred as an important factor in gas exchange in red blood cell, which can associate with hemoglobin and play a key role in CO_2 exchange [14]. Complete elimination of carbonic anhydrase from hemoglobin is not attained by
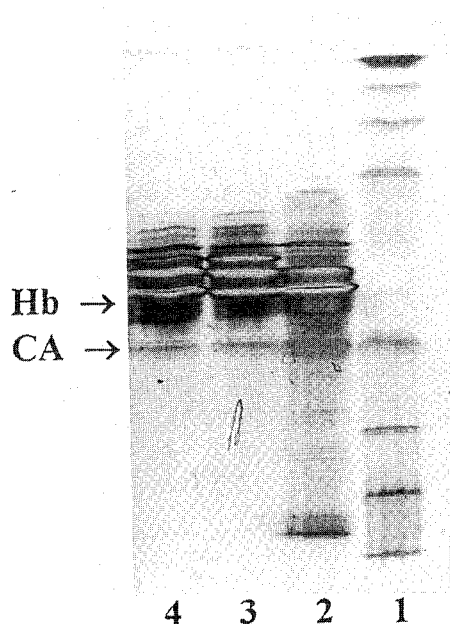
FIGURE 3   Isoelectric focusing pattern of hemoglobin detected by silver staining. Lane 1
represents standard pI kit (Pharmacia); lane 2 represents hemolysate prior to
the first extraction stage; both lane 3 and 4 represent Hb purified by
Aqueous two-phase system. There are many bands standing for different
types of human hemoglobin (e.g. Hb F, Hb B, ..and etc.) located above pI 7.
Carbonic anhydrase (CA) has pI of 6.5 around.

previous methods [3,6]. According to the sensitivity of IEF (silver staining), the amount of
the residual impurity was less that 0.1%. The analysis of phospholipids by the
chromatograms (Fg.4), indicates that only a trace of them still exist after by ATPS
method. The amount of residual phospholipids were less than 2% by comparison with the
area integration of chromatography of hemolysate. The oxygen affinity of purified Hb is
examed by the oxygen dissociation curve in Fig.5. The P_{50} of Hb is somewhat lower than
that of the fresh hemolysate.

DISCUSSION

Aqueous two-phase system features many advantages in the downstream of
fermentation industry. The system replaced the expensive filtration unit and partially

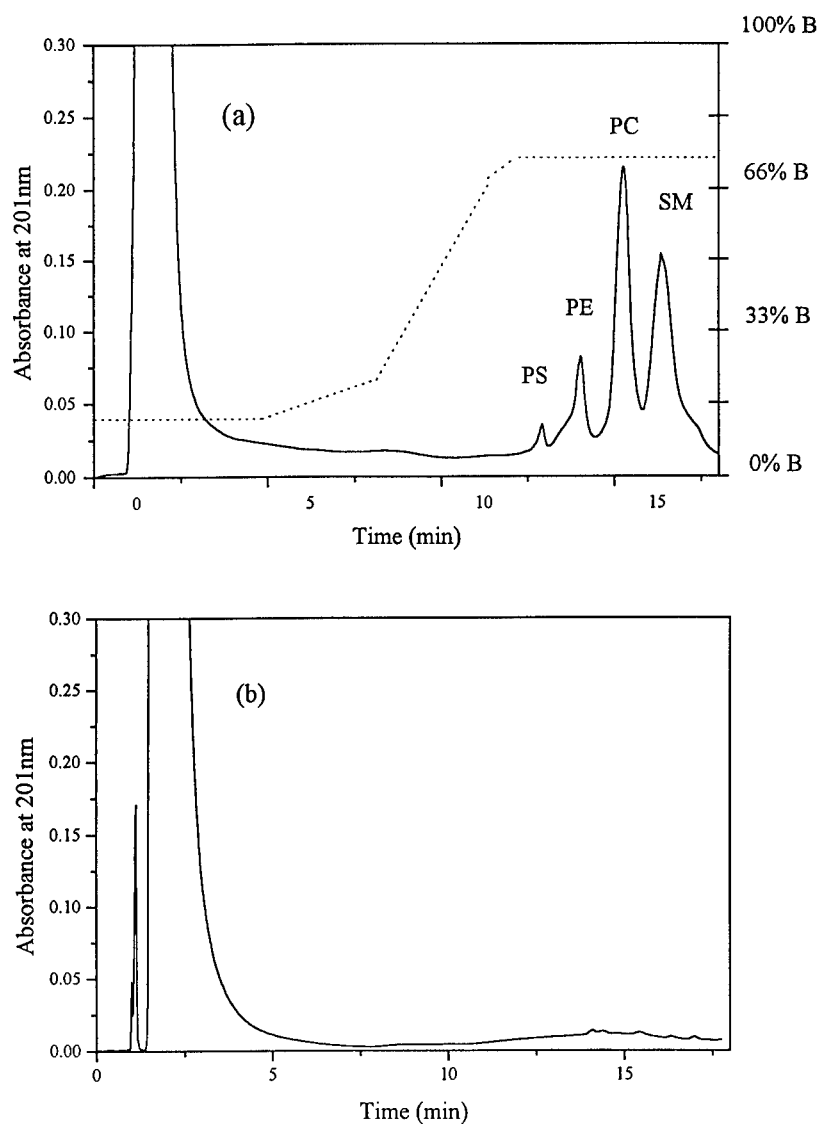FIGURE 4   The chromatography of phospholipids of red blood cell membrane analyzed
by HPLC. (a) represents the sample extracted from hemolysate prior to the
first extraction stage; (b) represents the sample extracted from Hb purified
by Aqueous two-phase system. There was a solvent front within first five
minutes in each plot. Neutral lipids would be eluted before phospholipids and
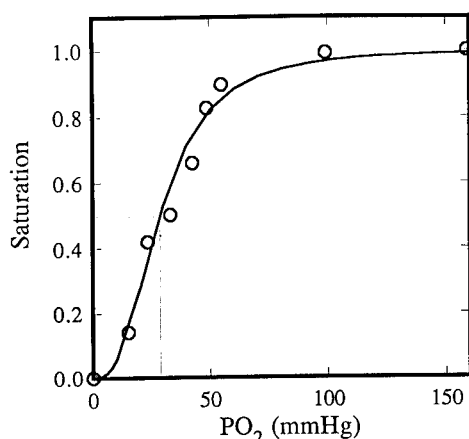would not interfer the analysis of phospholipids.

FIGURE 5   The oxygen dissociation curve of purified Hb by ATPS. P_{50} of Hb is 29.53
mmHg comparing to 33 mmHg of fresh hemolysate in this experimental
condition; the hill coefficient is 2.64. The concentration of Hb is 1.1 mg/ml
in 0.25 M phosphate buffer (pH 7.4) at 37°C [17].

purified the target protein in the broth mixture. Hemoglobin, like those intracellular
engineered proteins, may be properly purified by ATPS. In this study, hemoglobin was
separated from cell membranes in the first extraction and purified from other impurity
proteins in the second extraction. Also, it required short operation time to complete the
overall process. In addition, the materials for formation of phase system are inexpensive
and nontoxic for human being. Moreover, the ATPS required only low speed centrifuge
rather than the high speed centrifuge. Therefore , total investment and running cost are
lower than other separation methods. Purification of highly pure hemoglobin may be
achieved easily by this method in a common lab.

Recently, the development of engineered proteins accelerated the study on production
of *in vitro* hemoglobin. There have been a few of studies on human recombinant
hemoglobin for large-scale production in *E.coli* or *S.cerevisiae* [15,16]. If such technology
become a useful process, the hemoglobin-containing broth from fermentation tanks will be
comprised of much more complicated composition than whole blood, but aqueous two-
phase system will still be applicable. Because the function and characteristics of ATPS is
generally powerful for downstream treatment in most of fermentation industry.

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CONVENIENT METHOD TO PURIFY HEMOGLOBIN

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ABSTRACT

A convenient method to purify Hb solution from outdated RBC has been established for the starting material of Hb-based blood substitutes. To prevent MetHb formation during the procedure, Hb in RBC was carbonylated in advance. Then RBC was mixed with organic solvent for hemolysis and centrifuged for removal of stroma. The resulting SFHb solution was heated at 60°C and generated precipitates were removed out by centrifugation. The purity of Hb (25 g/dl) was confirmed by SDS-PAGE. IEF and oxygen binding property of the Hb solution also guaranteed its purity and no denaturation of Hb. This method is applicable to large scale production of the purified Hb for the starting material of Hb-based blood substitutes.

INTRODUCTION

One key technology to develop Hb-based blood substitutes *(1-6)* is a simple and large scale purification method of conc. Hb from outdated RBC. The requisite is to remove out stroma components, water soluble proteins other than Hb. Considering the disadvantages of the conventional methods (*e.g.* low concentration, low purity, long purification time), we have established a new purification method by taking the advantage of excellent stability of carbonylhemoglobin (HbCO). Mixing with organic solvents and heating were applied *(7, 8)* to remove stromata and proteins other than stable HbCO, respectively.
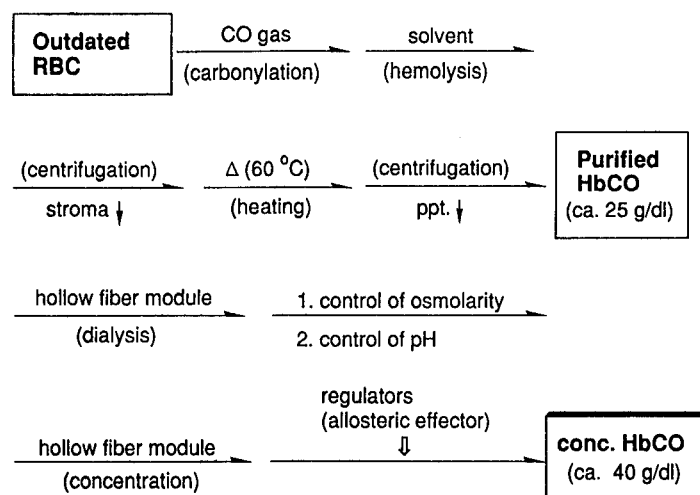
MATERIALS AND METHODS

Purification of Hb (SCHEME 1):   An outdated RBC solution was diluted with an eq. amount of saline. Air in a flask was replaced with a CO gas, and the flask was shaken for 90 sec to convert HbO_2 to HbCO. RBC was washed twice with saline by centrifugation ($\geq 1,000g, \leq 10$ min) *(Process 1)*. The conc. RBC solution ([Hb]: 25 g/dl) thus obtained was mixed with 0.2-fold CH_2Cl_2, shaken for 3 min and then centrifuged ($\geq 1,900g, \leq 15$ min). A Hb solution was separated as a top layer. After repeating the procedure again, residual CH_2Cl_2 in the Hb solution was removed out at ca. 20 torr at 40 °C in dark *(Process 2)*. Finally, the Hb solution was heated at 60 °C for 1 h in dark. The generated aggregates were removed out by centrifugation ($\geq 1,900g, \leq 20$ min), and the purified and conc. Hb solution was obtained *(Process 3)*.

Adjustment of solution conditions: The purified Hb solution was dialyzed against pure water by using a hollow fiber module (*e. g.* Clirans C15W, Terumo Co.). The Hb solution is ultrafiltrated by the same hollow fiber module, or an ultrafiltration system (TOSOH Co. Ltd.) with filters (M_r cutoff: 30,000).

RESULTS AND DISCUSSION

Carbonylation of Hb (Process 1): One characteristic point is to stabilize Hb in RBC by carbonylation. The carbonylation was promoted by dilution of the viscous RBC with an eq. volume of saline and shaking under a CO atmosphere. No direct contact of CO gas to Hb prevents denaturation of Hb. The diluted RBC solution is concentrated easily to [Hb] of 25 g/dl by gentle centrifugation. This process, which comprises dilution, carbonylation and centrifugation, serves RBC washing.

Hemolysis and removal of stromata by organic solvent (Process 2): The homogenized RBC/CH_2Cl_2 was centrifuged to give a SFHb solution as a top layer. The components of the middle layer between the SFHb solution and CH_2Cl_2 layers were analyzed by SDS-PAGE (FIG. 1). Membrane proteins such as ankyrine, glycophorin were detected in the layer, but not in the purified Hb solution. The band at 16,000 was Hb subunit and remained in the middle layer. The membrane phospholipids were effectively removed out and the percentage of residual phospholipids measured by HPLC were less than 0.2 % after the second

```
┌──────────┐  CO gas            solvent
│ Outdated │ ─────────────  ─────────────
│ RBC      │ (carbonylation)   (hemolysis)
└──────────┘
```

```
                                              ┌──────────┐
 (centrifugation)    Δ (60 °C)   (centrifugation)  │ Purified │
────────────────  ──────────── ──────────────  │ HbCO     │
   stroma ↓        (heating)        ppt. ↓       │(ca. 25 g/dl)│
                                              └──────────┘
```

```
 hollow fiber module      1. control of osmolarity
────────────────────  ────────────────────────
     (dialysis)           2. control of pH
```

```
                          regulators
                        (allosteric effector)
                                ⇓             ┌──────────┐
 hollow fiber module    ─────────────────────  │ conc. HbCO│
────────────────────                          │(ca. 40 g/dl)│
    (concentration)                           └──────────┘
```

SCHEME 1 The procedure of the purification of Hb from outdated RBC and adjustment of solution conditions.

treatment (TABLE I). The great advantage is that concentration of SFHb is almost the same as that of the washed RBC solution (25 g/dl). In all the purification utilizing hypotonic hemolysis *(9, 10)*, the concentration is less than 5 g/dl since a large amount of a hypotonic solution is added. Residual solvent in the Hb solution was evaporated to less than 0.1 ppm which was confirmed by gas chromatography. CH_2Cl_2 is chemically more stable than $CHCl_3$ and easier to be removed because of its low boiling point. Diethylether and hexane are also effective and gave high removal efficiencies. IEF pattern of the SFHb solution showed no change in p*I* and intensities of Hb variants in comparison with those of conventional hypotonic hemolysis.

Heat treatment (Process 3): DSC thermograms of HbO_2 and HbCO showed endothermic points at 62.2 °C and 77.7 °C, respectively. It indicates that HbCO is more stable than HbO_2. Any denaturation of HbCO was not caused during the heating at 60 °C, where virus inactivation in pharmaceutic products often performed. The proteins other than HbCO were denatured to form aggregates rapidly within 60 min and were easily removed out by gentle centrifugation. The SDS-
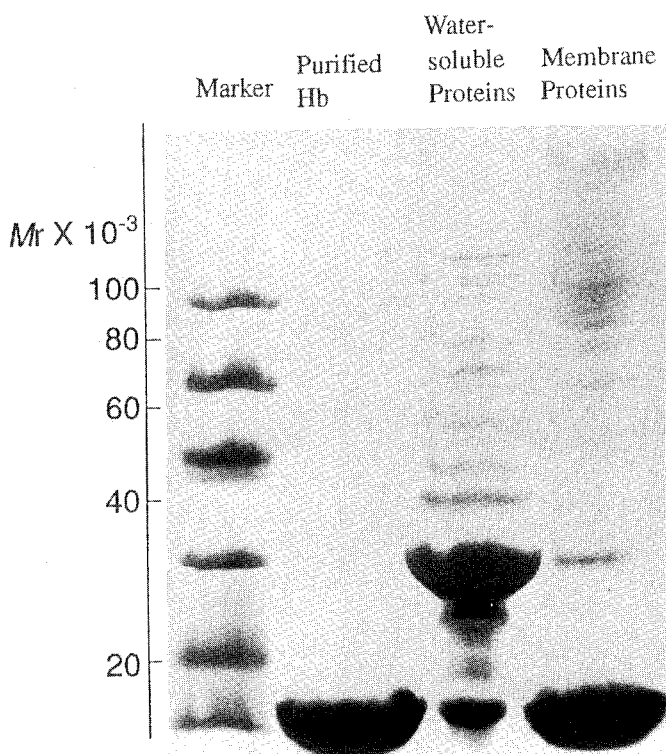
FIGURE 1 SDS-PAGE patterns of the components in the fractions separated by the purification procedure (performed with PhastSystem; Marker: electrophoresis calibration kit for low Mw.; Pharmacia). The gel was stained with PhastGel Silver Stain Kit.

PAGE pattern of the precipitates showed many proteins such as carbonic anhydrase (CA; $Mr = 30,000$)(FIG. 1). It is noted that percentage of MetHb decreased after heating. This suggests that MetHb would be also denatured by heating.

HbCO solution heated at 60°C for 12 h also showed no change in IEF pattern. The oxygen binding curve of the Hb solution after decarbonylation was identical with that of a SFHb solution purified by the conventional method. These results supported that the quaternary structure of Hb was preserved after the whole procedure.

TABLE I Specification of the purified Hb solution.

[Hb]	ca. 25	(g/dl)
yield (from washed red cell)	80	(%)
Purity	99.95	(%)
metHb	< 1.0	(%)
oxygen binding property	no change	
residual phospholipid[1]		(%)
phosphatidylserine (PS)	0.2	
phosphatidylethanolamine (PE)	0.1	
phosphatidylcholine (PC)	0.2	
sphingomyelin (Sph)	0.1	
residual organic solvent	< 0.1	(ppm)
the time required	3	(hr)
(with pasteurization)[2]	12	(hr)

1) $\dfrac{([\text{Lipid}] / [\text{Hb}])_{\text{Hb}}}{([\text{Lipid}] / [\text{Hb}])_{\text{Red Cell}}} \cdot 100$

2) heating procedure is prolonged to 10 hr.

Adjustment of solution condition and conversion of HbCO to HbO$_2$: After dialysis of the purified Hb against pure water by using a hollow fiber module, pH and osmolarity are adjusted to appropriate values. Ultrafiltration of the solution to 45 g/dl is easily carried out because the lipids and denatured proteins other than Hb, which increase the solution viscosity or clog the filter, were removed out completely. To control the oxygen binding properties, allosteric effectors such as pyridoxal 5'-phosphate are added to the solution. The Hb solution (ca. 40 g/dl) is applied to the starting materials of Hb-vesicles *(2-5)*. HbCO is easily decarbonylated to regenerate HbO$_2$ by exposing the solution to oxygen and visible light. One effective method is to expose the Hb vesicles to oxygen through a hollow fiber module with illuminating visible light at low temperature.

    In conclusion, Hb was stabilized by carbonylation in RBC to prevent denaturation during the purification procedure. Stroma components and water-soluble proteins were effectively removed out by mixing with organic solvents and heating, respectively. Hb concentration of the purified solution was 25 g/dl, which was the same as that of the conc. RBC solution. The whole process is very

simple and completed within 3 h with a high yield (80 %). It is applicable for mass production of purified Hb. After adjustment and concentration of the Hb solution, this is encapsulated in lipid vesicles to produce Hb-vesicles.

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PRODUCTION OF PEG-MODIFIED BOVINE HEMOGLOBIN: ECONOMICS AND FEASIBILITY.

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ABSTRACT

Bovine hemoglobin has many advantages as a blood substitute: a) it's ready availability; b) it's low cost; c) it's oxygen carrying capacity; and d) the ease with which it can be modified with polyethylene glycol (PEG) to improve its pharmacokinetic profile. This study investigates the potential of PEG-modified bovine hemoglobin as a cost-effective blood substitute.

INTRODUCTION

Hemoglobin-based red blood cell substitutes have been an elusive goal. Bovine hemoglobin, which under physiological conditions has an oxygen affinity similar to that of blood, is free of human viral contamination, is relatively inexpensive and readily available, would seem to be an ideal candidate as an oxygen-carrier, but progress toward its use as a blood substitute has been hindered by the unavailability of sufficient high-quality product for testing. This study investigates the potential of PEG-modified bovine hemoglobin as a cost-effective, high-quality, blood substitute.

METHODS

A single lot of bovine hemoglobin, prepared from aseptically drawn blood from Enzon's controlled herd of cattle, was used for all procedures. The

657

hemoglobin was isolated, modified with polyethylene glycol, formulated into a final product at 6%, $\pm 0.1\%$, distributed into sterile Terumo bio-bloodbags, and stored at 4°- 6°C.

The stability of this product was assessed over a 16 week period. Samples were tested at 1 week intervals for 12 weeks, and 2 week intervals thereafter. All samples were taken in triplicate and tested for pH and hemoglobin/methemoglobin concentrations. The percent yields per process were based on grams of bovine hemoglobin recovered, from collection of red blood cells to final product. One unit of final product is defined as 250 ml at 6% $\pm 0.1\%$.

Several pharmacokinetic parameters of this product were investigated in rats.

Purity analysis of bovine hemoglobin

An HPLC profile was done on a Zorbax (GF-450) size exclusion column using a Waters Maxima 820 chromatography unit. Isoelectric focusing, native (8% pre-cast Tris-glycine gel from Novex) and SDS polyacrylamide gels were run. The native gels were run in duplicate, at 2 different concentrations.

Purity of final product

The purity of our final product was determined by capillary electrophoresis run on the 270HT system produced by Applied Biosystems, Inc.

Endotoxin determination

Endotoxin levels were determined by the Molecular Devices System, utilizing a Thermomax Microplate reader for the Limulus Amebocyte Lysate (LAL) assay. The samples for endotoxin determination were taken at the end of the 16 week testing period.

Methemoglobin/hemoglobin concentration

The Radiometer/Copenhagen OSM3 Hemoximeter, with a PRS 12 Alpha printer, was used to determine the percentages of methemoglobin and hemoglobin.

RESULTS

Processing and Purity

A new, Class 100,000 clean-room facility, now available, enables all processing to be completed in a controlled environment kept at 4-6°C. Bulk yields of 33% are standard, with approximately 200 units processed per run. A unit is defined as 250ml, at 6% ±0.1%.

Stability studies on this lot of PEG-modified bovine hemoglobin over a four month period, showed no loss in hemoglobin concentration from the 6% starting level; pH exhibited a slow, but continuous, rise from 7.4 ±0.1 to 7.8 ±0.1, remaining within physiologic limits; methemoglobin levels remained low, with maximum levels reaching 5-6%; and endotoxin levels were 0.3 EU/ml (Fig.1).

The average time to process one lot of PEG-modified bovine hemoglobin was 5 days. On day one, the 45% loss in recovery is due to the process of squeezing the hemoglobin from the red blood cells. This procedure prevents an accumulation of impurities in the isolated hemoglobin and yields a hemoglobin, ready for polyethylene glycol modification, that is >99% pure. After this initial loss, all processing steps result in yields of 90% or better. Our processing of bovine red blood cells results in hemoglobin exhibiting 99.25 to 99.5% purity, depending on the method of measurement (Fig.2). HPLC (Fig.3) and capillary electrophoresis (CE) (Fig. 4) of this native hemoglobin results in single peaks, with no evidence of impurities. The purity of the polyethylene glycol-modified hemoglobin (PEG-Hb) was determined by HPLC (Fig.5), CE (Fig. 6), and native gels (Fig. 7), and visually confirms shifts in retention times and molecular weight resulting in a modified hemoglobin with 99.5% purity. Capillary electrophoresis, using Applied Biosystems, Inc. Model 600 Data Analysis System has proven to be very useful in providing information not available using conventional size-exclusion chromatography.
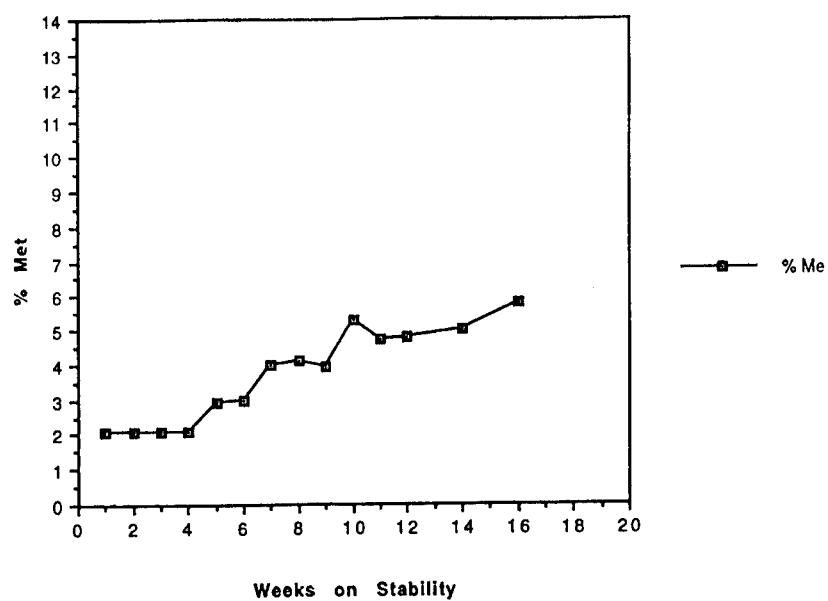
FIGURE 1. STABILITY OF PERCENT METHEMOGLOBIN IN PEG-HB.

Method of Determination		
SEC-HPLC	Single Peak	99.5% Pure
SDS-PAGE	Single Band	
IEF		PI = 7.2 + 0.1
CE	Single Peak	99.25% Pure

FIGURE 2. PURITY OF ISOLATED HB.
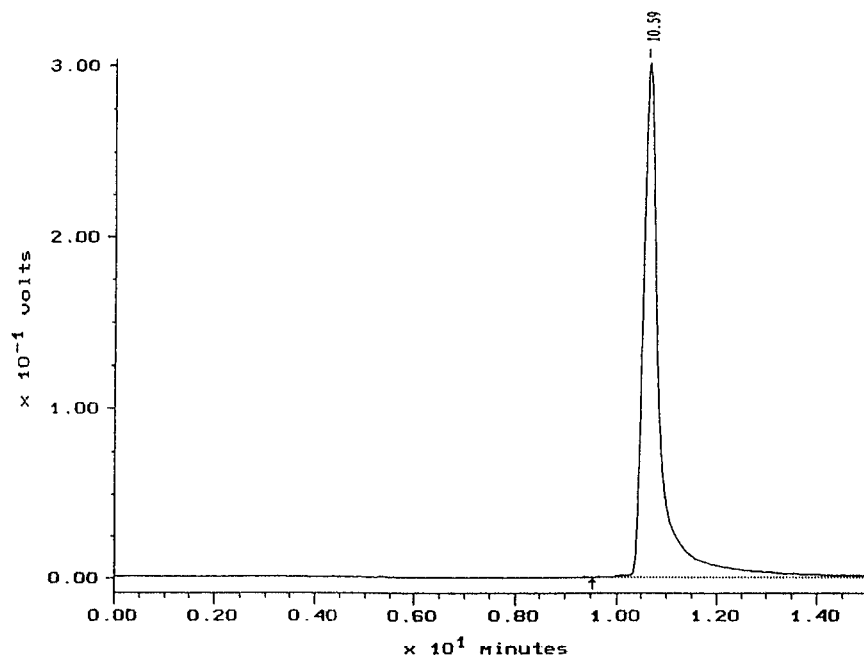
FIGURE 3. HPLC PROFILE - NATIVE HEMOGLOBIN.
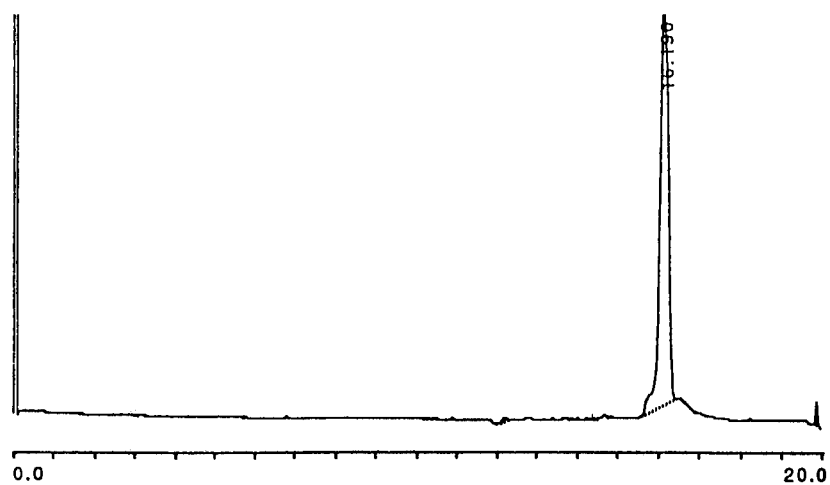
Analysis: Channel A

Peak No.	Time	Type	Height(μV)	Area(μV-sec)	Area%
	12.230	N	1164	6883	0.437
1	16.190	N	122586	1560415	99.257
	19.915	N	7721	4794	0.304
Total Area				1572092	99.998

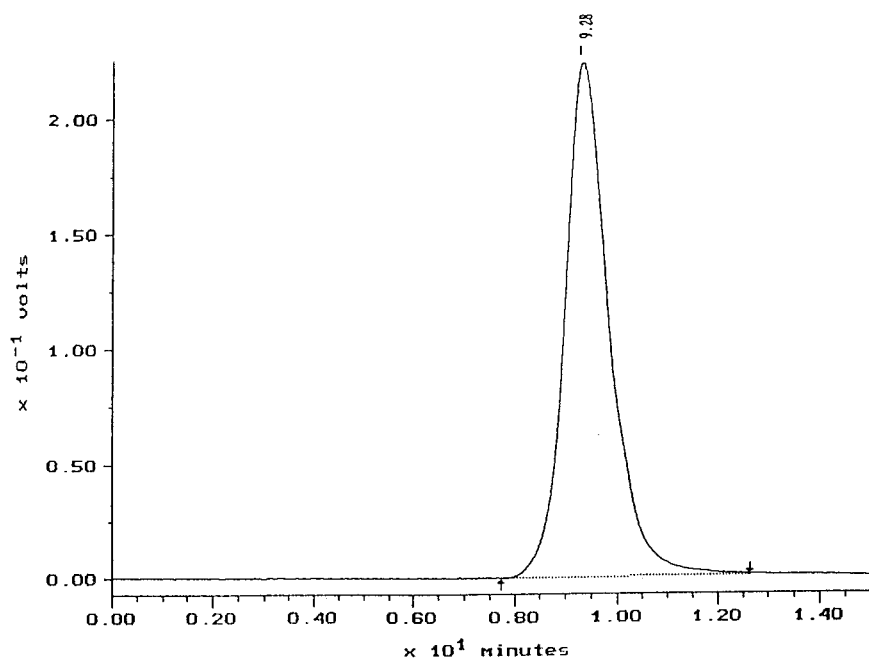FIGURE 4.   CE PROFILE OF ISOLATED NATIVE BOVINE HB.

FIGURE 5. HPLC PROFILE - ENZON PEG-HB.

The percent area for native hemoglobin, 99.25% (Fig. 4), coincides with that
for the modified hemoglobin (Fig. 6), indicating that 99.508% of the isolated
hemoglobin is PEG-conjugated during the reaction sequence. Both samples
were analyzed using the same protocol. Using native gel, this lot demonstrates
a clear distinction between the native and modified bovine hemoglobin (Fig.
7). These results have proved to be reproducible from one processed lot to
another.

Oxygen Transport Capacity

An exchange transfusion model and a hypovolemic shock model were used to
investigate the oxygen transport capability of PEG-Hb as compared to Ringer's
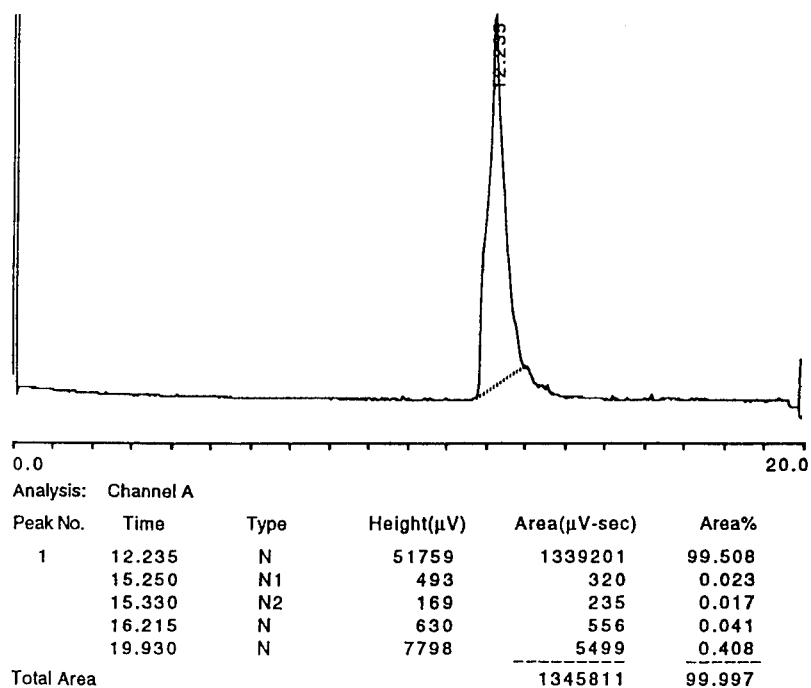
Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	12.235	N	51759	1339201	99.508
	15.250	N1	493	320	0.023
	15.330	N2	169	235	0.017
	16.215	N	630	556	0.041
	19.930	N	7798	5499	0.408
Total Area				1345811	99.997

FIGURE 6. CE PROFILE OF PEG-HB.

lactate solution and autologous blood. Using the Stern-Volmer equation, the tissue oxygen concentration was measured by a phosphorescence decay method in which the quenching of phosphorescence by oxygen is calculated for the free oxygen in tissue, given the quenching constants of the metallophorphyrin used [1].

Results of a 30% (blood volume) exchange transfusion in rats indicate that there is no decrease in muscle oxygen tension with the use of PEG-Hb, while Ringer's lactate as replacement fluid results in a 70 - 90% decrease in muscle tissue oxygen (Fig.8).
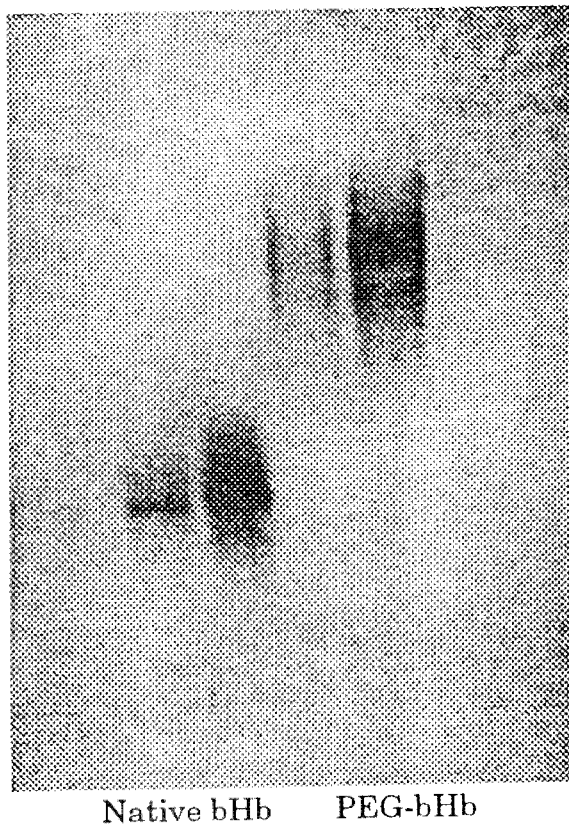
Native bHb   PEG-bHb

FIGURE 7. NATIVE GEL OF NATIVE AND PEG-HB.

In the rat hypovolemic shock (30% blood loss) model, rats resuscitated with PEG-Hb demonstrated a rapid recovery to normal tissue oxygen levels, delivering oxygen as well as, or better than, autologous blood, whereas, animals resuscitated with Ringer's lactate solution evidenced a marked drop in muscle tissue oxygen (Fig.8).

Type of Transfusion	% Recovery to Normal Tissue Oxygen Tension
30% Exchange Transfusion with Ringers Lactate	10% - 30%
30% Exchange Transfusion with Pegylated Bovine Hemoglobin	100% - 130%
30% Hypovolemic Shock, Resucitation with Ringers	5% - 30%
30% Hypovolemic Shock, Resucitation with PEG-Hemoglobin	90% - 130%
30% Hypovolemic Shock, Autologous with Whole Blood	80% - 100%

FIGURE 8.   RESULTS OF OXYGEN DELIVERY IN RATS USING BOVINE PEG-HB.

Animal	% E.T.	Hemoglobin uria up to Day 2 (mg/ml)	Kidney		Kidney	
			2D	7D	2D	7D
KN30-1	30	0	OK		OK	
KN30-2	30	0		OK		OK
KN30-3	30	0		OK		OK
KN50-1	50	0	OK		OK	
KN50-2	50	0		OK		OK
KN50-3	50	0		OK		OK
KN70-1	70	0	OK		OK	
KN70-2	70	0		OK		OK
KN70-3	70	0		OK		OK

FIGURE 9.   EXCHANGE TRANSFUSION.

Animal #	Dosage (ml)	Hemoglobinuria up to Day 2 (mg/ml)
MJ5-1	5	0
MJ5-2	5	0
MJ5-3	5	0
MJ10-1	10	0
MJ10-2	10	0
MJ10-3	10	0
MJ10-4	10	0

FIGURE 10. HEMOGLOBINURIA STUDY IN RATS USING PEG-HB
BOLUS INJECTION.

Hemoglobinuria

Rats exchange transfused, up to 70% blood volume (Fig.9), or low to high
bolus injected (12.5 - 25ml/kg) (Fig.10), with PEG-modified hemoglobin
demonstrated no hemoglobinuria.

CONCLUSIONS

Polyethylene glycol-modified bovine hemoglobin has been shown to be 99.5%
pure, stable for up to 16 weeks, can deliver oxygen to tissues at levels
equivalent to autologous blood, and shows no evidence of hemoglobinuria
when infused into the rat.

Enzon's herd is controlled for variables of feed, climate and health
maintenance. This control provides a ready source, free of AIDS and hepatitis
B viral contamination, of fresh red blood cells which are of consistant quality,
and which yield hemoglobin that can be easily processed for use as a red blood
cell substitute. Additionally, this herd provides an economical alternative to
outdated blood or recombinant forms of hemoglobin.

Our new, Class 100,000 processing/purification facility allows production of 200 units in a 5 day cycle.

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HUMAN HEMOGLOBIN CONJUGATED TO CARBOXYLATE DEXTRAN AS
A POTENTIAL RED BLOOD CELL SUBSTITUTE.
I. FURTHER PHYSICO-CHEMICAL CHARACTERIZATION.

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ABSTRACT

In a previous paper [1], we described the preparation of a conjugate by reaction of benzene tetracarboxylate-substituted dextran (dex-BTC) with oxyhemoglobin. The first biological experiments carried out on animals [2] showed that a solution of this type of product could be regarded as an oxygen-carrying fluid.

The physico-chemical properties of this conjugate have been further characterized in view of its potential application in clinical experiments. Its molecular size distribution and the fraction of intramolecularly cross-linked hemoglobin were determined by elution on various types of chromatographic columns. Moreover the conjugate's oxygen-binding properties were studied in the presence of effectors and of Cl^- ions, and the results showed that the allosteric site and the Val 1α residues of conjugated hemoglobin were occupied by dex-BTC. Finally, the fractions obtained after gel filtration exhibited a similar P_{50} whatever their molecular sizes. So far, all the results obtained are compatible with the use of the conjugate solution as a blood substitute.

INTRODUCTION

It is now a well established fact that solutions of purified human hemoglobin (Hb) are not capable of replacing blood, first because Hb binds oxygen too tightly,

and second because it is quickly lost from the vascular system ; the first drawback is caused by the lack of the natural effector and the second one by the vascular and renal clearance of dimers. Many Hb derivatives have been described, the aim of the chemical modifications of the protein being to improve simultaneously its oxygen affinity and its intravascular persistence. Recently, intramolecularly cross-linked Hbs have been prepared [3-4] but while the stabilized tetrameric structure did not diffuse through the kidney, its plasma retention only increased by a 2-4 factor compared with unmodified Hb ; this value is still too low for use in blood substitution and therefore some supplementary chemical modifications of Hb aiming to increase its size by polymerization or polymer fixation, must be considered [5].

Our strategy was to prepare, in a single step, a modified Hb presenting both large size and low oxygen affinity, by linking macromolecular effectors to native oxyHb. One of the macromolecular effectors used was dextran substituted with benzene tetracarboxylate groups (dex-BTC). Some preliminary properties of the resulting conjugate have already been described [1]. This paper gives the results of further physico-chemical characterization.

MATERIALS AND METHODS

The dex-BTC-Hb conjugate solution was prepared and purified as previously described [1] : 1 l containing about 70 g of modified Hb was obtained from the reaction of 37 g of dex-BTC with 74 g of oxyHb. Inositol hexaphosphate (IHP) was purchased from Sigma (USA), AcA 34 and AcA 202 Ultrogels from Sepracor-IBF (France), TSK SW G4000 column from Touzart et Matignon (France). The Mono-S column and Sephacryl S100 were obtained from Pharmacia (Sweden).

Oxygen-binding measurements

The oxyhemoglobin and methemoglobin concentrations were estimated from the solution absorbances at three wavelengths 630, 576 and 560 nm. The oxygen dissociation curves were determined using two techniques depending on the temperature. At 37°C the measurements were carried out on a Hemox Analyzer (TCS Corp., South Hampton, PA). P_{50} and VO_2 were determined from these curves. VO_2 is the O_2 volume that can be unloaded at 37°C, pH 7.4, by 1 g of Hb, native or modified, between 100 torr (arterial pressure) and 40 torr (venous

pressure). At 25°C the oxygen-binding curves were determined by a spectrophotometric method, using a tonometer, as previously described [6].

RESULTS

Chemical characterization of the conjugate solution

After reaction of dex-BTC with oxyHb, the solution was diafiltered on a 100,000 cut-off membrane in order to remove unreacted dex-BTC. The concentration of this unreacted polymer in the final conjugate solution was determined by anthron on the supernatant obtained after precipitation of conjugated and native Hb by trichloroacetic acid. This concentration was 2.5 ± 0.5 g/l.

The percentage of unmodified Hb was determined by two methods : the first one was gel filtration on a TSK SW G4000 column (Figure 1) and the second one, cation exchange chromatography on a Mono-S column with a 10 mM sodium malonate buffer, pH 5.7, LiCl gradient (not shown). Both methods gave a similar result of 15 ± 1 % of unmodified Hb with regard to total Hb.

The percentage of unmodified dimers was calculated from the gel filtration profile obtained in a dissociating medium (1 M $MgCl_2$) [7] on a Sephacryl S100 column (Figure 2). It was found to be 16 ± 0.5 % with regard to total dimers.

Distribution of molecular weights

The SEC-LALLS method, which consists in size exclusion chromatography coupled to, on one hand, a spectroscopic or refractometric detector and, on the other hand, a low angle laser light scattering detector, was used to determine the different average molecular weights of the conjugate [8]. Unfortunately this method, which is very useful for statistically substituted polymers, turned out to be unsuitable in the case of the heterogeneous polymer-Hb conjugates because the output given by the classical detector could not be related to the concentration in species of a given molecular size. Therefore, to further characterize the conjugate size distribution, the size exclusion chromatography column was calibrated with model proteins. Thus, the conjugate elution profile obtained with UV detection (Figure 1) shows that the major part of the species has a Stokes radius of between about 30 Å and 60 Å (to be compared to that of human serum albumin which is of 29.2 Å).
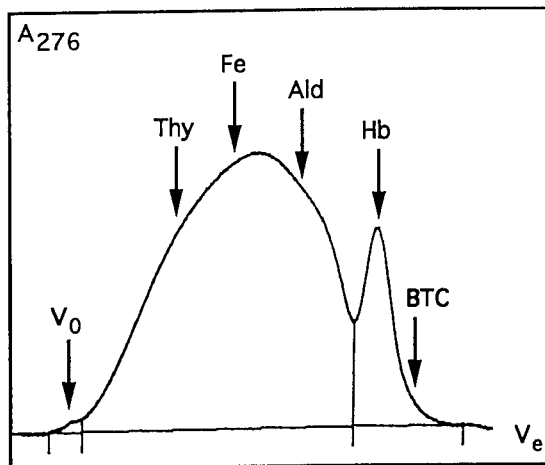
FIGURE 1 : Gel filtration profile of the dex-BTC-Hb conjugate.
TSK SW G4000 ; 0.05 M phosphate buffer pH 7.2 ; 0.7 ml/mn. V_0 corresponds to the void volume of the column. The other arrows give the elution volumes of model compounds : Thy R_h = 62.8 Å ; Fe R_h = 54.6 Å ; Ald R_h = 38.8 Å (R_h = hydrodynamic/Stokes radius).
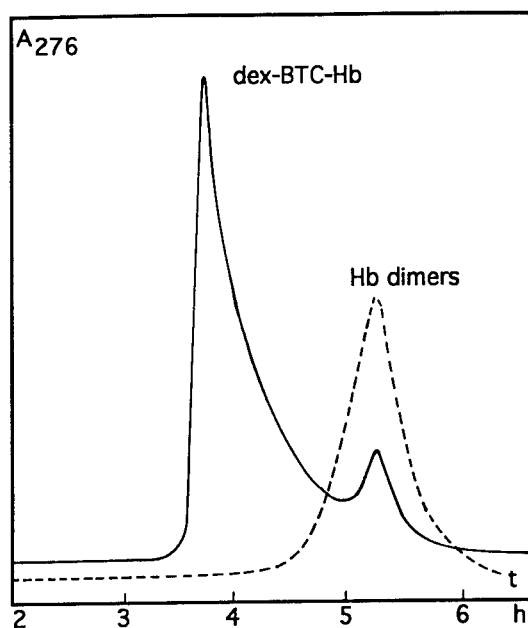

FIGURE 2 : Gel filtration profile of the conjugate in a dissociating medium.
S100 Sephacryl (100 x 2.5 cm) ; 0.1 % Tris buffer pH 7.2, 1 M $MgCl_2$, 47.8 ml/h ;
—— conjugate, ---- native Hb.

	37°C, pH 7.4			25°C, pH 7.2	
	P_{50} torr	P_{50}^a torr	VO_2^b ml/g	P_{50} torr	P_{50}^c torr
blood	27	-	0.32	-	-
native Hb	14	20	0.07	3.5	45
dex-BTC-Hb	21	21	0.31	8.8	9.9

TABLE I : Oxygen-binding properties of the conjugate solution
[a] in the presence of 10 mol of pyridoxal phosphate per mol of total Hb. [b] ml of O_2 unloaded between 100 and 40 torr per g of total Hb. [c] in the presence of 10 mol of IHP per mol of total Hb. pH 7.4 : 0.05 M Bistris buffer, 0.14 M NaCl, 40 mM glucose. pH 7.2 : 0.05 M Tris buffer

Oxygen-binding properties

Table I gives some oxygen-binding characteristics of the conjugate solution at 37°C, pH 7.4 and at 25°C, pH 7.2, in the presence or in the absence of effectors.

Figure 3 shows the effect of Cl^- ions (a) and of pH (b) on the conjugate P_{50}. An increase in the Cl^- concentration provoked an increase in the P_{50} of native Hb, while no effect was observed on that of conjugated Hb. The Bohr effect was respectively -0.30 and -0.50 for conjugated and native Hb.

Finally, the P_{50} values of the conjugate fractions obtained by gel filtration separation on AcA 34 Ultrogel were determined. The results are reported in Figure 4 and Table II and they show that the oxygen-binding properties are almost identical, whatever the molecular size of the fraction considered.

DISCUSSION

This kind of polymeric conjugate, which is easily prepared from oxyHb, presents many advantages for transfusion purposes. First, it contains less than 5 % of methemoglobin, and despite the fact that its P_{50} is not as high as that of blood it can release the same amount of oxygen between 100 and 40 torr at 37°C, pH 7.4. Second, its P_{50} is low whatever the molecular size of the fraction, which means that even if there is some in-vivo modification of the molecular size distribution,
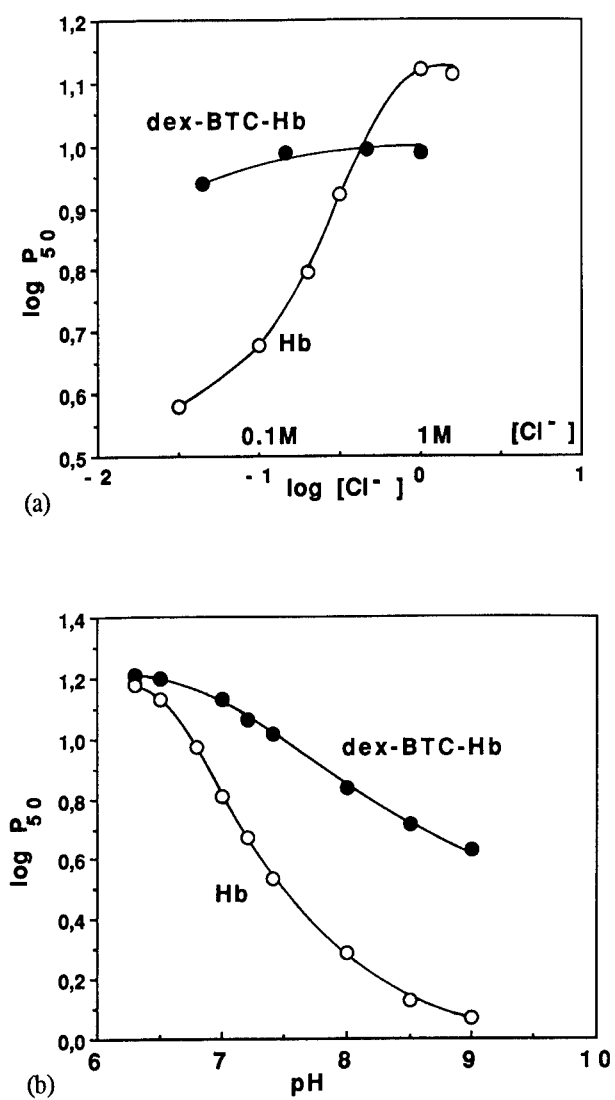
QUELLEC ET AL.

(a)

(b)

FIGURE 3 : Effect of Cl$^-$ ions (a) and pH (b) on the conjugate P$_{50}$.
P$_{50}$ measured at 25°C, pH 7.2, as in Table I.
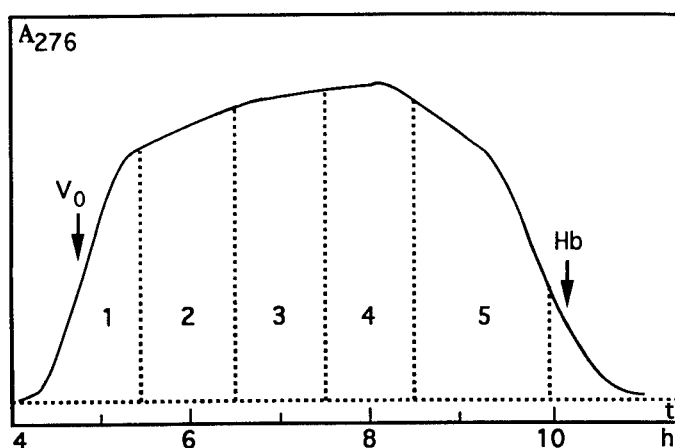
FIGURE 4 : Gel filtration profile of the conjugate on AcA 34 Ultrogel.
60 x 2.5 cm ; 0.05 M Tris buffer, pH 7.2, 27ml/h.

fraction $N^{\circ a}$	1	2	3	4	5	dex-BTC-Hb
$\%^b$	10.5	20.0	20.8	23.2	23.1	
P_{50} torr	9.3	9.4	9.5	8.9	8.5	8.8

TABLE II : P_{50} as a function of the molecular sizes of the conjugate fractions
[a] corresponding to the N° of Figure 4 ; [b] with regard to total Hb. P_{50} measured at
25°C, 0.05 M Tris buffer pH 7.2.

the oxygen release should not change a lot. On the other hand the major part of the
conjugate has a hydrodynamic radius larger than 30 Å and consequently it was not
filtered through the kidney membrane when injected into guinea pigs[2]. The low
molecular weight species evidenced by size exclusion chromatography in a
dissociating medium, corresponds to unmodified dimers and their quantity is
directly related to that of unmodified tetrameric Hb evidenced in Figure 1.

Finally, the lack of effect of IHP and of Cl⁻ ions on the conjugate P_{50} proves
that first the Hb allosteric site is occupied by the polymeric reagent (probably

through an amide linkage between dex-BTC and Val 1β which has the most reactive amine function in this cleft), and second that the Val 1α residues are also substituted, which incidentally explains the decrease in the absolute value of the Bohr effect.

So far, all these results are compatible with the use of this conjugate solution as a blood substitute, and the results of further in-vivo experiments confirm this possibility [9]. Finally it is now possible, by filtrating the conjugate mixture on a cation exchange chromatography column, to decrease the percentage of unmodified Hb to 2-3 % without modifying the general properties of the conjugate. Consequently, urinary loss was no longer observed when the conjugate solution was injected into guinea pigs .

ACKNOWLEDGEMENTS

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STRUCTURAL AND OXYGEN-BINDING PROPERTIES OF DIVINYL-SULFONE-REACTED BOVINE HEMOGLOBIN AS A FUNCTION OF PROTEIN LIGATION STATE AND REACTANT CONCENTRATION

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ABSTRACT

Bovine hemoglobin (HbBv) was reacted with divinyl sulfone (DVS) at 2-4°C and pH 7.4 for 24 h. Two procedures were employed: (1) low concentration (conc.) of oxygenated HbBv (ligand O_2) and low molar ratio of DVS/oxyHbBv (25 ml of 8% oxyHbBv, 0.01 M DVS); (2) low conc. of deoxygenated HbBv (no ligand) and low DVS/deoxyHbBv molar ratio (25 ml of 8% deoxyHbBv, 0.01 M DVS). The nonpolymerized purified products of procedures 1 and 2 were designated oxyHbBv-DVS and HbBv-DVS. Utilizing a high conc. solution of oxyHbBv-DVS and a relatively high molar ratio of DVS/oxyHbBv-DVS (25 ml of 15% oxyHbBv-DVS, 0.03 M DVS) it was possible to aerobically polymerize the modified hemoglobin (procedure 3). Similarly it was possible to anaerobically polymerize HbBv-DVS (25 ml of 15% HbBv-DVS, 0.03 M DVS)(procedure 4). The polymerized products of procedures 3 and 4 were designated oxyPoly HbBv-DVS and Poly HbBv-DVS. The four isolated products were characterized structurally using SDS-PAGE and gel-permeation HPLC and functionally employing a Hemox-analyzer at 37°C, pH 7.4 and 0.15 M Cl⁻. Both oxyPoly HbBv-DVS (P_{50} = 13 mm Hg, n = 1.1) and Poly HbBv-

DVS (P_{50} = 61 mm Hg, n = 1.6) were shown to be mixtures of intermolecularly-crosslinked hemoglobins. OxyHbBv-DVS (P_{50} = 13 mm Hg, n = 1.3) was shown to be an intramolecularly-crosslinked 64 kDa material, whereas HbBv-DVS (P_{50} = 52 mm Hg, n = 1.9) was found to be an intramolecularly-modified 64 kDa derivative, but not an intramolecularly-crosslinked one. The results indicate that intramolecular crosslinkage is achieved only in the presence of oxygen, while high P_{50} and n values are obtained when the reaction is performed in the absence of the oxygen ligand.

INTRODUCTION

Hypothetically, the reaction of hemoglobin (Hb) with the bifunctional reagent divinyl sulfone (DVS), can progress by way of one, or sometimes two, major steps. First, the covalent modification of the Hb occurs (Fig. 1a), and then a crosslinkage may happen. This crosslinkage can be intramolecular (Fig. 1b) and/or intermolecular (Fig. 1c). We have shown recently that the anaerobic reaction of bovine Hb (HbBv) with DVS results in a noncrosslinked intramolecularly-modified derivative and that by employing a high molar ratio of DVS to this derivative it was possible to effect anaerobic intermolecular crosslinkage. The relatively low oxygen affinities and methemoglobin contents of both the noncrosslinked intramolecularly-modified material (HbBv-DVS) and the modified polymerized material (Poly HbBv-DVS) persuaded us to examine their potential as blood substitutes [1,2].

Some initial studies on the reaction of HbBv with DVS were done in aerobic conditions using oxygen as the Hb ligand (E. Ilan, unpublished data). Under these conditions, we always obtained derivatives possessing very high oxygen affinities and therefore, not suitable for use as blood substitutes. It became obvious to us that the ligation state of HbBv during its reaction with DVS affects the ultimate oxygen affinities of the reaction products as well as their structural features.
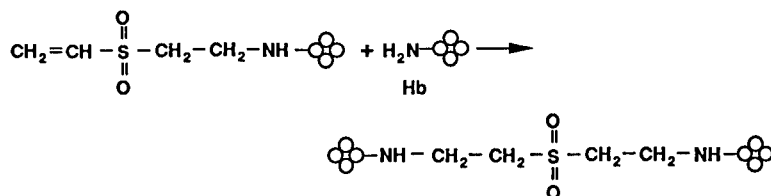
The purpose of this report is to evaluate the influence of ligation state and reactant concentration (conc.) on the structural and the oxygen-binding properties of DVS-reacted HbBv.

$$CH_2=CH-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH=CH_2 + H_2N-\text{Hb} \longrightarrow CH_2=CH-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_2-CH_2-NH-\text{Hb}$$

(a) modification

$$CH_2=CH-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_2-CH_2-NH-\text{Hb} \longrightarrow NH-CH_2-CH_2-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_2-CH_2-NH$$

(b) intramolecular crosslinkage

$$CH_2=CH-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_2-CH_2-NH-\text{Hb} + H_2N-\text{Hb} \longrightarrow$$

$$\text{Hb}-NH-CH_2-CH_2-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_2-CH_2-NH-\text{Hb}$$
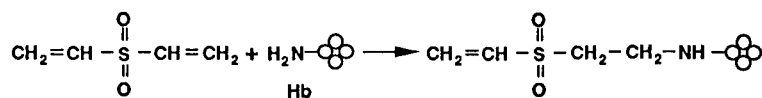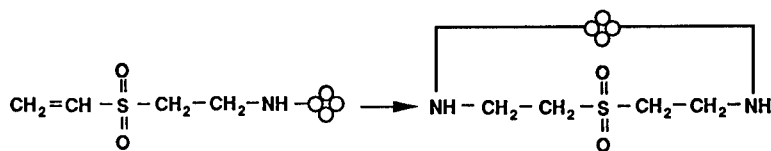
(c) intermolecular crosslinkage

FIGURE 1.   Schematic presentation of possible reactions between DVS and hemoglobin.

MATERIALS AND METHODS

HbBv, isolated and purified as described elsewhere [2], has been reacted with DVS at 2-4°C in 0.05 M sodium phosphate buffer (pH 7.4) for 24 h and then quenched for 18 h by adding lysine-HCl solution to a final conc. of 0.15 M.   Two different reaction procedures (combinations of protein ligands and reactants concs.) were employed : low conc. of oxygenated HbBv (ligand O_2) and low molar ratio of DVS/oxyHbBv (25 ml of 8% oxyHbBv, 0.01 M DVS (25 µl)) (procedure 1); low conc. of deoxygenated HbBv (no ligand) and low DVS/deoxyHbBv molar ratio (25

ml of 8% deoxyHbBv, 0.01 M DVS (25 μl))(procedure 2). The nonpolymerized products of procedures 1 and 2 designated oxyHbBv-DVS and HbBv-DVS, respectively, were separated from the unreacted Hb by ion-exchange chromatography using DEAE-Sephadex A-50 column as described previously for the HbBv-DVS derivative [1,2].

Employing a high conc. solution of oxyHbBv-DVS and a relatively high molar ratio of DVS/oxyHbBv-DVS (25 ml of 15% oxyHbBv-DVS, 0.03 M DVS (75 μl)) it was possible to aerobically polymerize the modified Hb (procedure 3). Similarly it was feasible to anaerobically polymerize HbBv-DVS (25 ml of 15% HbBv-DVS, 0.03 M DVS (75 μl))(procedure 4). All other conditions of procedures 3 and 4, including reaction and quenching times, were identical to those utilized in procedures 1 and 2. The polymerized products of procedures 3 and 4 designated oxyPoly HbBv-DVS and Poly HbBv-DVS, respectively, were separated from the nonpolymerized material by gel-filtration using a Biogel P-100 column as described before for Poly HbBv-DVS [1,2].

The four isolated products were characterized structurally using sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol and gel-permeation high-performance liquid chromatography (HPLC). In addition, their oxygenation properties were assessed at 37°C in 0.15 M Tris-HCl buffer containing 0.15 M Cl⁻ (pH 7.4) with a model B Hemox-analyzer. Oxygen affinity and cooperativity were outlined in terms of oxygen pressure at half-saturation (P_{50}) and the Hill coefficient (n_{50}) given by the Hill plot at half-saturation. Details of the aforementioned techniques, used for the structural and functional characterization of the various reaction mixtures and isolated products, are given elsewhere [1,2].

RESULTS AND DISCUSSION

Effect of the ligation state on the structural properties

Gel-permeation HPLC plots of HbBv reaction mixtures and derivatives acquired from its reactions with DVS are portrayed in Fig. 2. Figure 3 shows typical
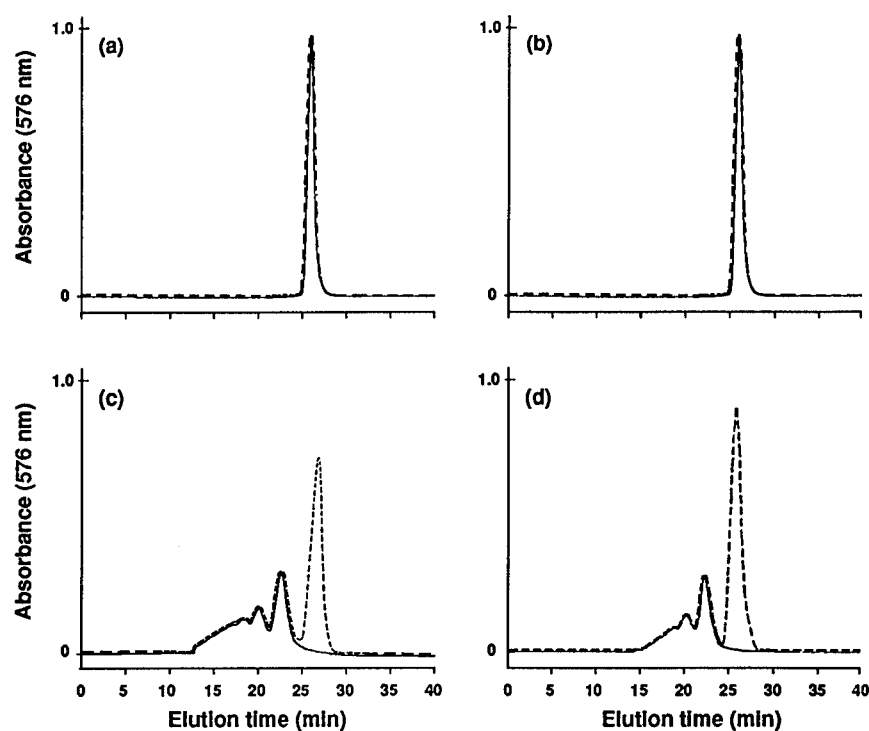
FIGURE 2. Gel-permeation HPLC of HbBv derivatives obtained from its reactions with DVS. (a) oxyHbBv-DVS 24 h reaction mixture (dotted line) and pure oxyHbBv-DVS (solid line). (b) HbBv-DVS 24 h reaction mixture (dotted line) and pure HbBv-DVS (solid line). (c) oxyPoly HbBv-DVS 24 h reaction mixture (dotted line) and isolated oxyPoly HbBv-DVS (solid line). (d) Poly HbBv-DVS 24 h reaction mixture (dotted line) and isolated Poly HbBv-DVS (solid line).

SDS-PAGE patterns of HbBv and of isolated derivatives obtained from its reactions with DVS. SDS-PAGE of the corresponding reaction mixtures was also performed (patterns not shown). From these Figs. it is evident that at low conc. of HbBv and low molar ratio of DVS/HbBv one is able to produce the nonpolymerized derivatives of oxyHbBv-DVS and HbBv-DVS. The single-peak HPLC chromatograms of these derivatives and of their reaction mixtures possess retention times identical to that of
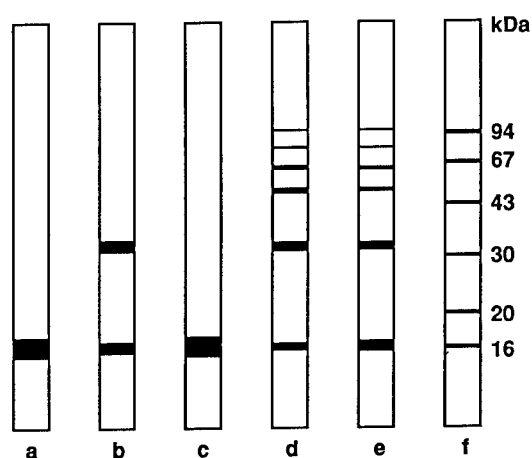
FIGURE 3. SDS-PAGE of HbBv and of isolated derivatives obtained from its reaction with DVS. (a) HbBv. (b) oxyHbBv-DVS. (c) HbBv-DVS. (d) oxyPoly HbBv-DVS. (e) Poly HbBv-DVS. (f) mixture of protein markers.

untreated HbBv, proving that both oxyHbBv-DVS and HbBv-DVS are indeed monomeric (64 kDa) nonpolymerized derivatives (Fig. 2a,b).

The effect of the ligation state on the structural properties of these derivatives is illustrated in Fig. 3 and Tables I and II. When the reaction is performed anaerobically (no ligand), the reaction mixture consists of 50% intramolecularly-modified Hb and 50% native Hb (Table I). The resulting purified derivative HbBv-DVS is 100% intramolecularly-modified uncrosslinked (intra- or intermolecularly) Hb (Table II). The absence of any 32 kDa band from the SDS-PAGE pattern of the pure HbBv-DVS proves the lack of intramolecular crosslinkage, while an ion-exchange chromatography single peak, slower than that of native HbBv (not shown), demonstrates 100% intramolecular modification. In contrast, when oxygen is used as a ligand the reaction mixture consists of 70% intramolecularly-crosslinked Hb and 30% native Hb (Table I). The ensuing purified derivative, oxyHbBv-DVS, is 100% intramolecularly-crosslinked (Table II). In SDS-PAGE, the intramolecular

TABLE I

Percentages of intramolecular modification, intra- and intermolecular crosslinkage in various reaction mixtures as a function of protein ligation state and reactants concentrations

Ligand	DVS Conc. (M)	Type, Vol. & Conc. of HbBv derivative used	Intra-molecular modification[a] (%)	Intra-molecular crosslinkage[a] (%)	Inter-molecular crosslinkage[b] (%)
oxygen	0.01	oxyHbBv 25 ml, 8%	0	70	0
no ligand	0.01	deoxyHbBv 25 ml, 8%	50	0	0
oxygen	0.03	oxyHbBv-DVS 25 ml, 15%	0	100	60
no ligand	0.03	HbBv-DVS 25 ml, 15%	100	0	50

Data in the Table are for reactions performed at pH 7.4 and 2-4°C for 24 h.
[a] Calculated from ion-exchange chromatography in conjunction with SDS-PAGE.
[b] Calculated from gel-permeation HPLC.

TABLE II

Percentages of intramolecular modification, intra- and intermolecular crosslinkage in various purified DVS derivatives of HbBv

Oxygen carrier	Mol. mass range [a] (kDa)	Intra-molecular modification[b] (%)	Intra-molecular crosslinkage[b] (%)	Inter-molecular crosslinkage[a] (%)
oxyHbBv-DVS	64	0	100	0
HbBv-DVS	64	100	0	0
oxyPoly HbBv-DVS	130 – 600	0	100	100
Poly HbBv-DVS	130 – 500	100	0	100

[a] Calculated from gel-permeation HPLC.

[b] Calculated from ion-exchange chromatography in conjunction with SDS-PAGE.

TABLE III

Oxygen-binding properties of HbBv and various purified DVS
derivatives thereof as compared with those of human whole blood

Oxygen carrier	P_{50} (mm Hg)	Hill coefficient	Δ % oxyHb (pO_2 100-40 mm Hg)
Human blood	26.5	2.6	25
HbBv	27	2.5	15
oxyHbBv-DVS	13	1.3	12
HbBv-DVS	52	1.9	37
oxyPoly HbBv-DVS	13	1.1	11
Poly HbBv-DVS	61	1.6	32

Measurements were performed in 0.15 M Tris-HCl buffer containing
0.15 M Cl⁻ at pH 7.4 and 37°C.

crosslinkage brings about the appearance of an additional band with a mobility
corresponding to 32 kDa indicating the formation of crosslinked dimers. After
purification, the relative amount of crosslinked dimers tended to approach the 50%
value (Fig. 3b) expected from complete intramolecular crosslinkage between two
subunits (Fig. 1b).

Effect of the ligation state on the oxygen-binding properties

The oxygen-binding properties of HbBv and its purified DVS derivatives are
presented in Table III.

The Table shows that the oxygen equilibrium curve for the non-liganded
manufactured HbBv-DVS is considerably right-shifted (P_{50} = 52 mm Hg) with respect
to native HbBv (P_{50} = 27 mm Hg), while that of the oxygen-liganded produced
oxyHbBv-DVS is rather left-shifted (P_{50} = 13 mm Hg). Oxygen-delivering capacities
calculated from the oxygenation curves of these derivatives between arterial (100 mm
Hg) and venous (40 mm Hg) oxygen partial pressures are shown to be 37% for

HbBv-DVS and 12% for oxyHbBv-DVS as compared to 15% for native HbBv and 25% for human whole blood (Table III). These results demonstrate that HbBv-DVS can serve as a potential source for oxygen carriers, possibly even hypothermic oxygen carriers [1], whereas oxyHbBv-DVS is not a good candidate for use as an oxygen transporter.

Effect of reactant concentration on the structural properties

Employing both high conc. of oxyHbBv and high molar ratio of DVS/oxyHbBv (25 ml of 15% oxyHbBv, 0.03 M DVS) we were able to effect substantial aerobic polymerization. The same was true for anaerobic polymerization using high conc. of deoxyHbBv and high DVS/deoxyHbBv molar ratio (25 ml of 15% deoxyHbBv, 0.03 M DVS). We did not usually use these reactions, since the isolated polymerized materials resulting from them contained 15 to 40% methemoglobin. In order to overcome this problem, routine use was made of purified oxyHbBv-DVS (procedure 3) and HbBv-DVS (procedure 4) instead of oxyHbBv and deoxyHbBv as previously described in Materials and Methods. In this regard, procedure 3 resulted in 60% and procedure 4 in 50% polymerization (Fig. 2c,d; Table I). The isolated material of procedure 3, oxyPoly HbBv-DVS, is a mixture of intramolecularly-crosslinked polymerized hemoglobins with a molecular mass range from 130 to ~600 kDa; that of procedure 4, Poly HbBv-DVS, is a mixture of intramolecularly-modified (but not intramolecularly-crosslinked) polymerized derivatives having similar molecular mass distribution (Fig. 2c,d; Fig. 3d,e; Table II).

Effect of reactant concentration on the oxygen-binding properties

Table III shows that the high concs. of reactants, while bringing about both aerobic and anaerobic polymerizations, did not cause drastic changes in the oxygen affinities. The oxygen affinity of oxyPoly HbBv-DVS (13 mm Hg) did not change at all compared to that of oxyHbBv-DVS, whereas the P_{50} value of Poly HbBv-DVS (61 mm Hg) increased somewhat with respect to that of HbBv-DVS (52 mm Hg).

The polymerization processes also effected moderate decreases in the Hill coefficients as well as in the oxygen-delivering capacities of the resulting materials (Table III).

CONCLUSION

The reaction of HbBv and DVS is characterized by the following dominant parameters: (a) intramolecular crosslinkage is achieved only in the presence of oxygen; (b) polymerization is effected at high concs. of HbBv or its appropriate derivative and relatively high molar ratios of DVS to this derivative; (c) high P_{50} and n values are obtained when the reaction is performed in the absence of the oxygen ligand.

ACKNOWLEDGEMENTS

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HUMAN HEMOGLOBIN ANAEROBICALLY REACTED WITH DIVINYL SULFONE: A SOURCE FOR OXYGEN CARRIERS

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ABSTRACT

Human hemoglobin (HbA) was reacted in anaerobic conditions with divinyl sulfone (DVS). The structural and oxygenation properties of the resulting chemically-modified product were studied in order to assess its potential as a physiological oxygen carrier. The reaction was carried out anaerobically at 25°C and pH 7.4 for 3 h. Quenching was performed with lysine-HCl solution and the resulting solution dialysed to remove unbound DVS and excess lysine. The product, designated Poly HbA-DVS, was characterized structurally using gel-permeation HPLC and SDS-PAGE and functionally employing a Hemox-analyzer at 37°C and pH 7.4. From gel-permeation HPLC it was estimated that about 60% of the starting material was polymerized, with a molecular mass range from 130 to about 500 kDa, and about 40% remained monomeric with a molecular mass of 64 kDa. The virtual absence of a 32 kDa band from the SDS-PAGE pattern of the last eluting HPLC peak and the oxygenation properties of this peak material ($P_{50} = 33$ mm Hg, $n = 2.2$; P_{50} very different from the ~15 mm Hg associated with native HbA solution) indicated that the monomeric (64 kDa) component was modified, but virtually noncrosslinked, within the tetramer. The product solution, Poly HbA-DVS, had a P_{50} of 35 mm Hg,

a Hill coefficient n of 1.8 and a methemoglobin content of 5-7%. This material has characteristics appropriate for an oxygen carrier, and can probably be used as such in perfusional and transfusional fluids.

INTRODUCTION

The anaerobic reaction of human hemoglobin with the bifunctional reagent divinyl sulfone (DVS) as described by Morris at al. [1] and by Bonsen et al. [2] results in intramolecular or intermolecular crosslinkage, depending on the reaction conditions.

In this account it is shown how one can use human hemoglobin (HbA) to achieve intramolecular modification with virtually no intramolecular crosslinkage and subsequently polymerize the hemoglobin in a one-step reaction using one chemical modifier-DVS. The product solution, designated Poly HbA-DVS, has a relatively low oxygen affinity and methemoglobin (MetHb) content and is therefore a potential cell-free oxygen carrier.

MATERIALS AND METHODS

Normal adult human hemoglobin (HbA), prepared as described by De Venuto et al. [3], was reacted with DVS at 25°C in 0.05 M sodium phosphate buffer (pH 7.4) for 3 h (50 ml of 13 g/dl deoxyHbA, 0.02 M DVS (100 µl)). Anaerobic conditions were continuously maintained by purging with nitrogen. Quenching was then carried out anaerobically for 1 h by adding 2.0 M lysine-HCl solution to a final concentration of 0.15 M. The resulting solution was dialysed thrice against 0.05 M sodium phosphate buffer (pH 7.4) in order to remove unbound DVS and excess lysine. The product solution, termed Poly HbA-DVS, was characterized structurally employing gel-permeation high-performance liquid chromatography (HPLC) and sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol. Its oxygen-binding properties were measured at physiological conditions (pH 7.4, 37°C) using a model B Hemox-analyzer. Oxygen affinity and cooperativity were characterized by the partial oxygen pressure at half-saturation (P_{50})

and the Hill coefficient (n_{50}) introduced by the Hill plot at half-saturation. A description of these techniques is available elsewhere [4,5]. Total hemoglobin and MetHb concentrations were measured with an IL 482 CO-Oximeter (Instrumentation Laboratory) calibrated for HbA extinction coefficients. The fact that the absorption spectrum of Poly HbA-DVS was virtually identical to that of HbA led us to select the Benesch et al. [6] procedure as a simple method for the estimation of the ferric hemoglobin content in this material. Colloid osmotic pressure (COP) was measured at 20°C with a WESCOR 4400 oncometer. For these measurements HbA and Poly HbA-DVS solutions were exchanged into 0.01 M phosphate-buffered saline (pH 7.4) and their concentrations adjusted to 12 g/dl.

RESULTS AND DISCUSSION

Structural properties of Poly HbA-DVS

Gel-permeation HPLC of typical HbA and Poly HbA-DVS preparations are presented in Fig. 1. From the figure it was estimated that about 60% of the native HbA was polymerized, with a molecular mass range from 130 to about 500 kDa (Fig. 1b fraction I), and about 40% remained monomeric with a molecular mass of 64 kDa (Fig. 1b fraction II).

Figure 2 shows SDS-PAGE patterns of Poly HbA-DVS and of its polymerized and nonpolymerized constituents, as compared with that of HbA. The absence of a significant 32 kDa band from the SDS-PAGE pattern of the nonpolymerized component of Poly HbA-DVS (Fig. 2b) and the oxygenation properties of this component material (P_{50} = 33 mm Hg, n = 2.2; P_{50} very different from the ~15 mm Hg associated with native HbA solution) indicate that this monomeric (64 kDa) component is modified, but virtually noncrosslinked within the tetramer. Consistent with the HPLC results (Fig. 1b fraction I) the electrophoretic pattern of the polymerized fraction (Fig. 2c) shows bands corresponding to a molecular mass of 16 kDa and multiples thereof, indicating that this fraction is made up of a mixture of heterogenous molecular mass polymerized HbA derivatives. The aforementioned observations strongly suggest that the anaerobic reaction of HbA with DVS proceeds
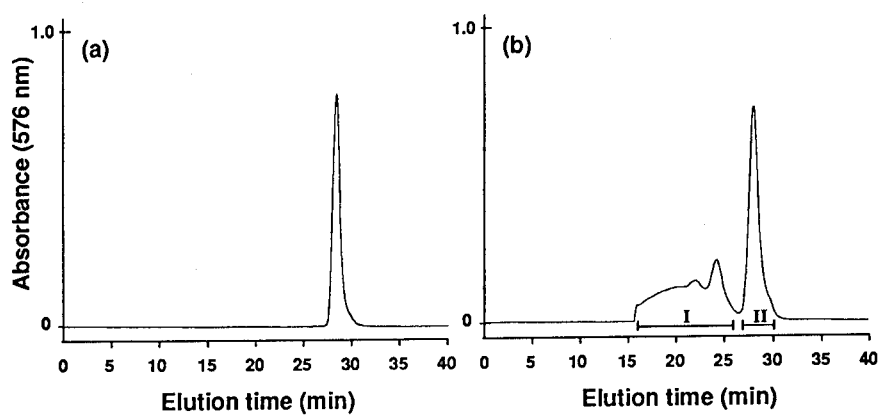
FIGURE 1. Gel-permeation HPLC of typical Poly HbA-DVS preparation as compared to that of native HbA. (a) HbA. (b) Poly HbA-DVS.
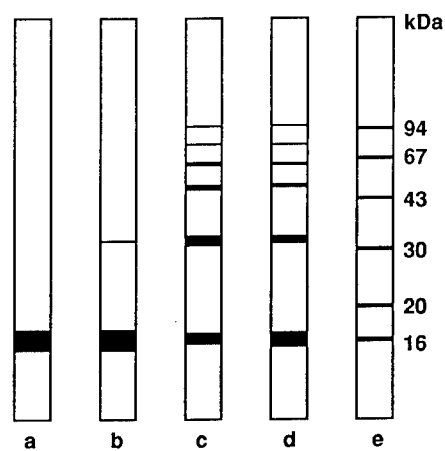
FIGURE 2. SDS-PAGE of HbA, Poly HbA-DVS and its polymerized and nonpolymerized components. (a) HbA. (b) The nonpolymerized component isolated from Poly HbA-DVS reaction mixture (fraction II of Fig. 1b).
(c) The polymerized fraction isolated from Poly HbA-DVS reaction mixture (fraction I of Fig. 1b). (d) Poly HbA-DVS. (e) Mixture of protein markers.
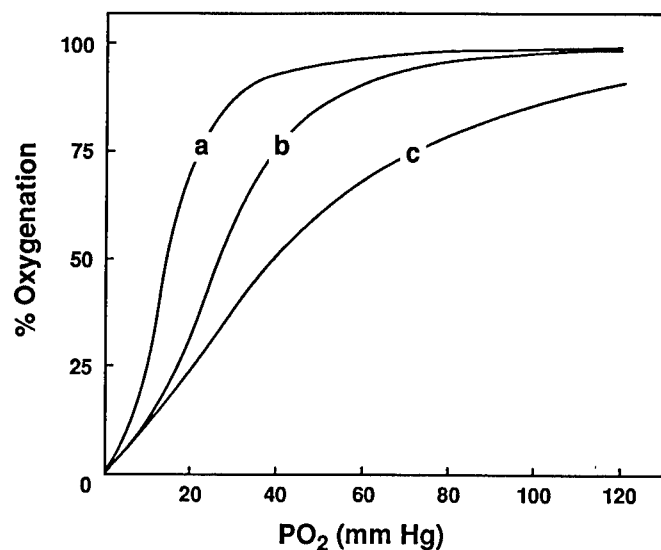
FIGURE 3. Oxygenation curve of Poly HbA-DVS preparation as compared to those of human blood and human HbA at 37°C and pH 7.4. (a) Human HbA. (b) Human whole blood. (c) Poly HbA-DVS.

through two major steps: First, the covalent modification of the hemoglobin which improves its oxygen-transporting properties, and then the intermolecular crosslinkage.

Oxygen-binding properties of Poly HbA-DVS

The oxygen equilibrium curve of typical Poly HbA-DVS preparation is presented in Fig. 3 in relation to curves obtained with human HbA and human whole blood. The conditions of these measurements were chosen to mimic the normal physiological situation, that is, pH 7.4 and 37°C. From Fig. 3 and Table I it can be seen that under these conditions Poly HbA-DVS has a P_{50} of 35 mm Hg and a Hill coefficient n of 1.8. Also evident from the Fig. and the Table is the fact that the P_{50} of Poly HbA-DVS at physiological pH and temperature has been increased compared to that of HbA and to that of human whole blood at the same conditions.

TABLE I

*Physicochemical properties of typical Poly HbA-DVS preparation
as compared with those of human whole blood and human HbA*

Physicochemical property	Human blood	Human HbA	Poly HbA-DVS
Hemoglobin conc. (g/dl)	14	12	12
COP (mm Hg)	25	45	27
MetHb conc. (% of total Hb)	0.5	2 – 3	5 – 7
pH at 37°C	7.4	7.4	7.4
Extent of polymerization[a] (%)	—	0	60
Molecular mass range[a] (kDa)	—	64	130 – 500
P_{50}[b] (mm Hg)	26.5	15	35
Hill Coefficient[b]	2.5	2.6	1.8

[a] Calculated from gel-permeation HPLC.
[b] Measurements were performed in 0.1 M sodium phosphate buffer
at pH 7.4 and 37°C.

CONCLUSION

High oxygen-delivering capacity in conjunction with low oncotic pressure render Poly HbA-DVS a promising oxygen-transporting erythrocyte substitute. By changing the concentration of HbA, the molar ratio of DVS/HbA and other conditions such as reaction time and temperature, it might be possible to change the proportions between the monomeric and the polymeric components thereby tailoring the material to various specific needs.

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RECOMBINANT HEMOGLOBIN A PRODUCED IN TRANSGENIC SWINE: STRUCTURAL EQUIVALENCE WITH HUMAN HEMOGLOBIN A

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ABSTRACT

Recombinant human hemoglobin A produced by coexpressing human α and β globin genes in swine, and purified from the lysate of transgenic swine has been subjected to detailed protein chemical analysis. These structural studies involving laser desorption mass spectrometry, separation of globin chains by RPHPLC, amino terminal sequence analysis of the isolated globin chains, the tryptic peptide mapping of the purified globin chains and the amino acid composition analysis of the purified tryptic peptides of globin chains have established the primary structural equivalence of the globin chains of the transgenic swine derived hemoglobin A with that of human hemoglobin A. These results demonstrate that the transgenic swine system correctly translates the human α and β globin m-RNA; carries out the correct cotranslational processing of globin chains, and does not introduce any unwanted post translational modifications into the mature chains. Thus, the transgenic swine expression system is an excellent approach for the production of HbA for developing an effective hemoglobin based oxygen carrier.

INTRODUCTION

Over the years there has been considerable interest in the potential of cell free hemoglobin as a substitute for red cells in oxygen delivery. The use of modified hemoglobin as a blood substitute suffers from various limitations [1, 2]. The oxygen affinity of cell free Hb is higher than that of the erythrocytes. Besides, the cell free Hb is susceptible to dissociation into $\alpha\beta$ dimers and this leads to a rapid clearance of Hb from the circulation and possible kidney damage. Moreover, the cell free Hb derived from human red blood cells may be contaminated with infectious agents such as hepatitis B and immunodeficiency virus. Finally, the supply of outdated blood as a source of Hb appears to be a limiting factor for producing an adequate supply of the blood substitute that will satisfy the anticipated current world wide needs.

Preparation of human Hb by the recombinant DNA technology can overcome most, if not all, of the limitations of using cell free Hb as blood substitute [3, 4]. Expression of human globin genes in heterologous systems provides an elegant approach to produce human pathogen free hemoglobin. Recombinant Hb could be designed by the techniques of genetic engineering in order to modulate the oxygen affinity and stability against dissociation. It is conceivable that this will eliminate the need for chemical modification of the Hb thus generated, one of the current successful approaches used to date. By the recombinant hemoglobin expression approach, a continous supply of Hb or rationally designed Hb free from the contamination of the blood borne human pathogens is virtually assured.

The recent advances in our understanding of globin gene regulation, particularly the discovery of upstream enhancer elements have enabled the development of transgenic animals expressing human Hb [5, 6]. This methodology has been translated as an approach that enables the large scale production of human pathogen free HbA in swine. The fact that the Hb expressed in transgenic swine will be located within the erythrocytes, further simplifies the large scale purification problems generally encountered with the hemoglobin A expressed in either yeast or bacterial systems.

The human HbA produced in transgenic swine has been shown to be chromatographically identical to the authentic HbA and also has an oxygen affinity

comparable to that of HbA [6]. However the structural equivalence of transgenic HbA to human HbA must be thoroughly established before this recombinant HbA could be considered for development into a blood substitute. Accordingly, the equivalence of the primary structure of the globin chains of the transgenic (recombinant) HbA with those of the α and β-chains of authentic HbA has been investigated and the results are presented here.

MATERIALS AND METHODS

Human HbA was prepared from the erythrocyte lysates by DEAE cellulose chromatography followed by a second CM-cellulose chromatography as described earlier [7]. The recombinant HbA (rHbA) was prepared by the chromatographic process developed recently for the purification of the protein from the stroma free Hb obtained from transgenic swines expressing human HbA [8]. The mass spectra of the recombinant HbA were obtained on a matrix-assisted laser desorption mass spectrometer constructed at The Rockefeller University and described elsewhere [9, 10]. The recombinant HbA was added to a solution of the laser desorption matrix material [sinapinic acid in 0.1 % trifluoroacetic acid/acetonitrile 2 : 1 (V/V)] to give a final concentration of hemoglobin of approximately $2 \mu M$. A small aliquot (0.5 μL) of this mixture was applied to the metal probe tip and dried with forced air at room temperature. The sample was then inserted into the mass spectrometer and analyzed, Bovine cytochrome C was used to calibrate the mass spectra. Globin from the hemoglobin sample was prepared by the acid acetone precipitation procedure. The Edman degradation of the globin chains was carried out using an ABI 477A sequencer. The PTH amino acid from each cycle was directly analyzed on a model 120A PTH analyzer attached to the sequencer. The tryptic digestion of the globin samples was carried out in 50 mM ammonium bicarbonate pH 8.0 at 37^0 for 5 to 7 hr. An enzyme to protein ratio of 1 : 100 was used. The tryptic peptides were separated using a linear gradient of 5 to 50% acetonitrile. An aquapore RP-300 column was used for these analyses. The amino acid analysis was carried out using a Beckman 123 analyzer using the post column derivatization with ninhydrin.

RESULTS

Molecular mass of the subunits of rHbA: The rHbA isolated from the transgenic swine erythrocytes using a modified large scale preparation procedure [8] showed a single band on isoelectric focusing with an isoelectric point corresponding to that of human derived HbA. This observation is consistent with the results of earlier studies in which the transgenic swine HbA was purified from swine Hb by DEAE cellulose chromatography, and had an oxygen affinity comparable to that of human HbA [6]. The molecular mass of the subunits of rHbA has been now determined by laser desorption mass spectrometry. The measured molecular mass for the two subunits of rHbA are 15,124.6 $\pm$ and 15868.2 $\pm$ daltons, respectively. The calculated values for the α and β-globin chains of human HbA are 15,126.7 and 15868.2 daltons, respectively. Thus the observed mass for the subunits of rHbA is identical within two mass units to the values calculated for the α and β globin chains of human HbA based on their amino acid sequence. These findings imply the correct cotranslational processing, and the lack of translational errors as well as post translational modification of globin chains when human globin genes are expressed in swine system.

Separation of the globin chains of rHbA: The RPHPLC analysis of the globin chains of rHbA on a Vydac C_4 column demonstrated that the heme as well as the two chains of rHbA have chromatographic characteristics identical to those from an authentic sample of human HbA. Besides the relative amounts of the two globin chains in rHbA (as reflected in the RPHPLC maps) suggests that the stoichiometry of the two chains corresponds well with that of the control sample of HbA. The two globin chains were isolated and repurified by a second RPHPLC for further structural studies.

Amino terminal sequence analysis of globin chains of rHbA: The globin chain of rHbA eluting at the position of α-globin has been sequenced for 40 cycles. The amino acid sequence data identified this peak as the α-globin, and established the correct processing of the amino terminus of the expressed chains and the absence of translational errors in this 40 residues sequence region. The globin chain eluting at the position of the authentic β-chain has been sequenced for 36 cycles. Again the amino acid sequence data are identical to that of an authentic β-globin sample. The results of the sequencing studies thus establish the correct cotranslational processing

of the amino terminal regions of human α and the β-globin genes expressed in the swine system.

Tryptic peptide mapping of α and β-globin chains of rHbA: The molecular mass data together with the amino terminal sequence data of the two chains of rHbA imply the correct cotranslational processing and the lack of post translational modification reactions. However, the molecular mass itself can not be considered as a clear cut evidence for the lack of any translational errors. With a view to establishing the primary structural equivalence of rHbA in regions beyond the amino terminal regions sequenced, the tryptic peptide mapping of the globin chains from rHbA, and the characterization of the individual tryptic peptide by amino acid composition of individual tryptic peptide have been undertaken. The RPHPLC tryptic maps of the α and β globin chains of rHbA are identical to the maps of the respective globin chains from a control sample of human HbA. The amino acid composition of the individual tryptic peptides from the globin chains of rHbA (the amino acid analysis have been completed for about 80% of the tryptic peptides from RPHPLC) have established the complete identity of the tryptic peptides of rHbA with that of the authentic sample. Thus the results presented here confirm the equivalence of the primary structure of α and β-globin chains of transgenic swine HbA with that of HbA.

DISCUSSION

The demonstration of the primary structural identity of the globin chains of rHbA with the globin chains of authentic human HbA establishes that the globin genes are expressed in the swine system without any translational errors, the cotranslational processing of the expressed chains proceeds in a fashion identical to that in the human system and the mature globin chains do not undergo post translational protein modification reactions. Additionally, the results of preliminary chromatographic studies of rHbA on a Mono S column have confirmed the chromatographic identity of the sample with that of the authentic human HbA. It is of interest to note here that using this chromatographic system, Adachi and his colleagues [11] have demonstrated the presence of a misfolded form of HbA in addition to the correctly folded HbA in their sample of rHbA expressed in the yeast

system and isolated by the DE-52 chromatographic system. The studies, thus, suggests the absence of such misfolded species in the rHbA sample from transgenic swine. Therefore, it may be concluded that the human globin chains expressed in swine are in an environment that permits their proper assembly into functional tetramer. Further detailed investigations of the conformational aspects and the functional properties of the transgenic swine HbA are underway to establish this aspect unequivocally.

The demonstration that the human HbA produced in transgenic swine is structurally equivalent to human HbA prepared from erythrocytes paves the way for the production of HbA and/or rationally designed mutant Hb with desired oxygen properties using this system. Thus, the transgenic swine system is an excellent and inexpensive source for the large scale production of authentic human Hb free of blood borne human pathogens that can be used for the preparation of a blood substitute.

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DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb™):
CHARACTERIZATION OF THE PROCESS AND THE
PRODUCT MANUFACTURED UNDER GMP REQUIREMENTS
FOR CLINICAL STUDIES

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ABSTRACT

Initiation of Clinical Trials in 1992 necessitated product manufacture under FDA Good Manufacturing Practice (GMP) requirements. To this end, all product components used were characterized to meet established requirements and processes and analytical methods were validated to confirm and document their utility and reproducibility. Manufacture of multiple 50+ liter lots of 10%g DCLHb under GMP requirements resulted in a pure and homogeneous product which reproducibly met a rigorous and complete set of product specifications. Final release testing of five consecutive GMP lots showed hemoglobin concentrations of 10.2 ± 0.2g%, pH of 7.33 ± 0.02 (37°C), methemoglobin concentrations of 3.2 ± 0.6%, degree of crosslinking of 99.8 ± 0.1% and a P_{50} of 32.4 ± 1.0 mmHg (37°C). The mean overall yield for production of these five lots was 55 ± 2%. Products were all endotoxin free as indicated by rabbit, cytokine and LAL testing (<0.06 EU/mL); a critical characteristic for a molecule which may bind endotoxin. All lots were sterile as indicated by compendial testing.

INTRODUCTION

Diaspirin Crosslinked Hemoglobin (DCLHB™) solutions have been previously introduced[1], and are currently the subject of clinical studies. These solutions have been extensively studied for safety and efficacy in numerous in vitro and in vivo systems.[2,3] The virus inactivation and removal in the production process of DCLHb has been reported previously.[4,5] The consistency and stability of DCLHb

701

prepared for preclinical studies was presented in the IVth ISBS Symposium in Montreal, 1991.[6,7]

In this article, five lots of DCLHb, produced under FDA Good Manufacturing Practice (GMP) requirements for clinical studies are characterized. In addition, parameters of the production process that were optimized and controlled through implementation of GMP requirements are identified.

MATERIALS AND METHODS

The process summary for production of DCLHb is shown in Figure 1. In this process the purified hemoglobin undergoes chemical modification in the deoxygenated form. The crosslinking reagent Bis(3,5-Dibromosalicyl) Fumarate is prepared by Baxter Healthcare Corporation.

RESULTS AND DISCUSSION

The scaleability, reproducibility and convenience of the chemical modification, a crosslinking process between alpha chains of hemoglobin, are demonstrated by the data collected from the five 50+ liter GMP lots (Table I) and by comparison of the results obtained in different manufacturing sites and different scales (Table II). The crosslinking process which is completed in five hours at all scales is essential for performing viral inactivation by heat. The final crosslinked hemoglobin is also more stable _in vitro_ and _in vivo_ and has more desirable oxygen dissociation characteristics than unmodified hemoglobin. The chemical modification process and the subsequent purification by heat treatment and ultrafiltration provided a degree of crosslinking of 99.8 ± 1% for the five GMP lots. The degree of purification of the lots produced under GMP requirements is further evidenced by the average quantities of the plasma protein IgG, and the erythrocyte enzyme carbonic anhydrase I, which are found in the final products on average at less than 0.8 and 2.1 ppm, respectively (Table I). The mean overall yield of 55 ± 2% for the five lots is indicative of lot to lot reproducibility of the process. The yield is also reproducible in multiple sites and scales as shown in Table II. The process times for other steps of production such as cell wash, hemoglobin purification, deoxygenation and the DCLHb purification in three manufacturing sites and two different scales are shown in Table II. This information demonstrates the scaleability and reproducibility of the DCLHb production process.

Red Blood Cells

            Cell Wash

            Cell Lysis

            Hemoglobin Purification:

                        •   **Ultrafiltration**

                        •   **Concentration**

                        •   **Diafiltration**

Purified Hemoglobin

            Deoxygenation

            Chemical Modification

            Heat Treatment/Viral Inactivation

Diaspirin Crosslinked Hemoglobin (DCLHb)

            DCLHb Purification:

                        •   **Ultrafiltration**

                        •   **Concentration**

                        •   **Diafiltration**

            Sterile Fill

Purified Diaspirin Crosslinked Hemoglobin

Figure 1
DCLHb™ Production Process Summary

The mean methemoglobin content of the five lots manufactured under GMP requirements was $3.2 \pm 0.6\%$. The methemoglobin values were determined after freezing the product to -20°C and then thawing the product. These values are approximately 1.0% on the day of manufacture. The quality of these solutions is also indicated by: (1) the nondetectable levels of endotoxin as determined by the limulus amebocyte lysate (LAL) test; (2) acceptable rabbit test results per USP; and (3) acceptable USP sterility testing results for all five lots.

Product quality and reproducibility were made possible through thoughtful consideration and definition of product and process requirements. The GMP

TABLE I. Physiochemical Parameters of Five Lots Produced Under GMP
Requirements

Parameter	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Total Hemoglobin (g/dL)	10.0	10.3	10.4	10.3	9.9
Methemoglobin (%)	4.1	2.9	2.3	3.6	2.9
pH at 37°C	7.31	7.33	7.32	7.36	7.32
Pyrogenicity (LAL, EU/ml)	<0.06	<0.06	<0.06	<0.06	<0.06
Pyrogenicity (Rabbit, USP)	Pass	Pass	Pass	Pass	Pass
P_{50} (mm Hg at 37°C)	31	32	33	32	34
Phospholipids (ppm)	0.2	0.2	0.1	N.D.	N.D.
Osmolality (mOsm/kg)	299	297	295	298	300
Crosslinking (%)	99.9	99.8	99.6	99.8	99.8
General Safety (USP/CFR)	Pass	Pass	Pass	Pass	Pass
Sterility (USP/CFR)	Pass	Pass	Pass	Pass	Pass
Carbonic Anhydrase I (ppm)	<2.9	<1.9	<1.9	<1.8	<2.0
IgG (ppm)	<2.5	<0.3	<0.3	<0.3	<0.3
Cytokine, IL-6 (ng/mL)	0.001	N.D.	0.002	0.014	N.D.

N.D. = None Detected

regulations, which apply to clinical product manufacture[8,9,10] contain a number of
elements for consideration (Table III). Careful control and monitoring of the
manufacturing environment, temperature, positive air pressure, air exchanges and
critical system components such as water for injection, steam, N_2 gas and
compressed air has an important impact on product and process quality. Routine
documented cleaning and sanitation of the facility and equipment is also important.
The numerous in-process evaluations and controls and component quality assurance
have also contributed to the production of a consistent, endotoxin-free and sterile
product for clinical studies. All activities and requirements associated with the
product manufacture and to support GMP compliance were included in Standard
Operating Procedures. Also key to product and process quality are the validation
studies performed to demonstrate the functionality, suitability and reproducibility of

TABLE II. Reproducibility of DCLHb™ - Comparison of Parameters of the Processes and the Products

Parameters	50-Liter Scale		5-Liter Scale
	Average 5 GMP Lots Round Lake, IL	Average 5 Lots Los Angeles, CA	Round Lake
Total Hb (g/dL)	10.2 ± 0.2	10.1 ± 0.1	10.0
Methemoglobin (%)	3.2 ± 0.6	2.7 ± 0.6	2.4
pH at 37°C	7.33 ± 0.02	7.42 ± 0.03	7.35
Pyrogenicity (LAL, EU/ml)	<0.06	<0.12	--
P_{50} (mm Hg)	32.4 ± 1.0	33.8 ± 0.4	--
Phospholipids (ppm)	0.1 ± 0.1	0.37 ± 0.26	--
Crosslinking (%)	99.8 ± 0.1	99.2 ± 0.7	99.6
Overall Yield (%)	55 ± 2	57 ± 2	55
Cell Wash Process Time (hrs)	8.0	8.0	8.0
Hb Purification Time (hrs)	10.0	12.0	12.0
Deoxygenation process Time (hrs)	6.5	6.5	6.0
Crosslinking Reaction Time (hrs)	5.0	5.0	5.0
DCLHb Purification Time (hrs)	10.0	12.0	10.0

activities. Table IV contains several areas where validation studies have been performed to demonstrate and document process efficacy. In these studies, performance standards were defined as closely as possible prior to study initiation. Multiple tests were then performed to evaluate conformance with requirements and reproducibility and to fine-tune routine operating limits. Data from these studies has further utility as a benchmark for process improvement and transfer of technology to other locations.

TABLE III. Good Manufacturing Practice Elements

Personnel	• Adequate Education/Background • Training
Components	• Requirements • Evaluation • Release
Equipment	• Use • Monitoring • Maintenance
Facilities	• Environmental Control • Cleaning • Monitoring
Process Control	• Processing Limits • In Process Testing
Release Testing	• Defined Requirements • Validated Methods
Procedure Systems	• For All Requirements and Activities
Documentation	• Comprehensive • Review Systems • Retention Times
Quality Control	• Independent Organization • Defined Responsibilities
Stability Program	• Ongoing

TABLE IV. Key Validation Activities

- Analytical Methods
- Filtration Processes
- Viral Inactivation
- Aseptic Filling
- Equipment Cleaning/Sanitization
- Sterilization Processes

CONCLUSION

Optimization of the manufacturing process concurrent with thorough identification and development of process controls and product specifications resulted in production of DCLHb for clinical studies. In addition, adherence to Good Manufacturing Practice requirements not only assured quality and reproducibility for production of DCLHb solutions at the 50 liter scale, but also provided a benchmark for process improvement and produced guidelines for the design of an economically feasible commercial scale process. Incorporation of GMP requirements to include extensive procedures and documentation will provide the data base needed to support product and process quality and equivalency upon scale up, and for marketing approval.

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ENZYMATIC PROTECTION FROM AUTOXIDATION
FOR CROSSLINKED HEMOGLOBINS

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ABSTRACT

The autoxidation rates of hemoglobins crosslinked between the α subunits (α99XLHb A) and between the β subunits (β82XLHb A) were reduced in the presence of catalase and/or superoxide dismutase. In the presence of catalase the rate for α99XLHb A decreased 2.3 fold and for β82XLHb A, 1.9 fold. Superoxide dismutase reduced the rate 1.6 fold for α99XLHb A and 1.8 fold for β82XLHb A. In the presence of both catalase and superoxide dismutase the rate of autoxidation decreased by 3.0 fold in α99XLHb A and 4.0 fold in β82XLHb A. The presence of catalase and superoxide dismutase or both in the crosslinked hemoglobin samples increases the autoxidation half-life of oxyhemoglobins. This suggests that crosslinked hemoglobins to be used as blood substitutes could be protected from oxidation in storage by these enzymes.

INTRODUCTION

Oxyhemoglobin in the body or in solution can autoxidize to methemoglobin. Free heme in solution oxidizes rapidly to hematin [1]. The resistance to oxidation of heme in hemoglobin and myoglobin is due largely to the coordination of the iron to the proximal histidine F8, the steric hindrance

from the distal histidine E7 of the globin chain, and the hydrophobic environment encapsulating the heme, retarding the binding of water and ions at the iron center [1]. These properties are further suggested by the picket fence synthetic heme models which were able to bind oxygen reversibly [1]. Crosslinking hemoglobin, however, may slightly perturb the bisphosphoglycerate binding cavity by pulling the F-helix toward the center in the β crosslinked hemoglobin [2]. This perturbation may change the heme pocket favoring the entry of radicals to the heme reaction center, which is consistent with the higher autoxidation rates observed for crosslinked hemoglobins [3].

The interest in α99XLHb A as a blood substitute arises from the facts that its oxygen binding properties are similar to those of whole blood [4,5] and that it is very stable [6]. However, its autoxidation rate was reported to be faster than either Hb A or β82XLHb A [3]. This will present problems for long term storage of crosslinked hemoglobins.

In the body, the level of methemoglobin remains constant at 1% due to the reduction of methemoglobin by the methemoglobin reductase, NADH-cytochrome b5 reductase system [7]. One of the requirements for the successful use of hemoglobin derivative as a blood substitute is the prevention of autoxidation. This could be achieved by the presence of reducing enzymes or antioxidants in the hemoglobin solution. They scavenge superoxide and hydrogen peroxide which accelerate the oxidation rate. This research investigates the prevention of hemoglobin autoxidation by superoxide dismutase and catalase.

EXPERIMENTAL PROCEDURES

Materials: The synthesis of bis(3,5-dibromosalicyl) fumarate was done according to the method of Zaugg et al. [8]. Hemoglobin was prepared according to the method of Dozy et al. [9] from blood obtained from Life Source blood bank. Superoxide dismutase and catalase were obtained from Sigma Chemical Co.

<u>Crosslinking Reactions:</u> The crosslinking reactions of hemoglobin with bis(3,5-dibromosalicyl) fumarate were carried out according to Walder et al. [2] for oxyhemoglobin (β82XLHb A) or Chatterjee et al. [4] for deoxyhemoglobin (α99XLHb A).

<u>Autoxidation Experiments:</u> The autoxidation of hemoglobin was monitored by the formation of methemoglobin according to the method of Tomita et al. [10] as modified by Yang and Olsen [3]. For samples containing catalase, the total enzyme units added prior to the start of incubation were 70,784 U/mL or 16,530 U/mM heme. A unit of activity of catalase is defined by the supplier (Sigma) as the amount of enzyme which will decompose 1 μmole of H_2O_2 per minute at pH 7.0 and 25°C while the H_2O_2 concentration falls from 10.3 to 9.2 μmoles/mL of reaction mixture. For samples containing superoxide dismutase, the total enzyme units added were 3,185 U/mL or 835 U/mM heme. The SOD was assayed by supplier (Sigma) using the method of McCord and Fridovitch [11]. In the experiments in which both catalase and SOD were present, the same amount of each enzyme described above was added. In all of these experiments, the catalase, when present, was 2.3% of the total protein, and the SOD, when present, was 1.1% of the total protein. Thus, the amounts of the added enzymes were not enough to attribute the results to the general effects of proteins as free radical scavengers. The k_{app} for hemoglobin autoxidation was determined as described by Yang and Olsen [3]. The $t_{1/2}$ was estimated from the k_{app} obtained for each sample.

RESULTS

The presence of catalase and SOD in the hemoglobin solution slows down the autoxidation rate of both uncrosslinked and crosslinked hemoglobins. Figure 1(A) shows the autoxidation of oxy Hb A in the presence of catalase, SOD or both. Figures 1(B) and (C) show similar data for oxy
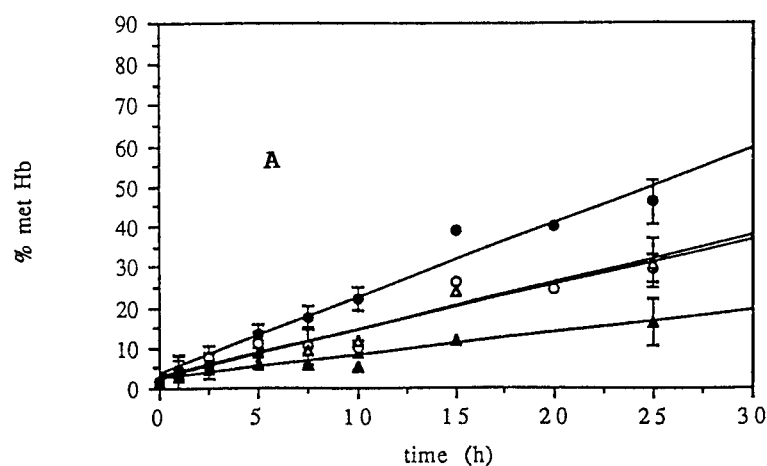
FIGURE 1: The formation of methemoglobin as a function of time for
hemoglobins. (A). Hb A (•), Hb A + catalase (○), Hb A +
SOD (△), Hb A + catalase + SOD (▲); (B). β82XLHb A (•),
β82XLHb A + catalase (○), β82XLHb A + SOD (△), β82XLHb
A + catalase + SOD (▲); (C). α99XLHb A (•), α99XLHb A
+ catalase (○), α99XLHb A + SOD (△), α99XLHb A +
catalase + SOD (▲).

β82XLHb A and α99XLHb A samples, respectively. When neither enzyme
was present the rate of methemoglobin formation was fastest in both
uncrosslinked and crosslinked hemoglobins. In the presence of either catalase
or SOD the autoxidation rate was reduced. When both enzymes were present
in the samples the reduction of autoxidation was more effective.

Table I shows the inhibition of autoxidation by SOD and catalase.
The combined enzymes decreased the autoxidation rate of β82XLHb A by 4
fold and α99XLHb A by 3 fold. The lesser inhibition in crosslinked
hemoglobins is probably due to the inherently faster autoxidation rate of
these species.
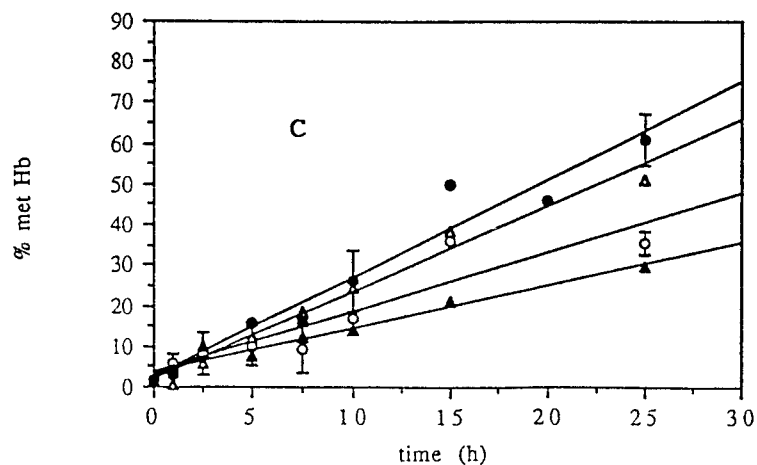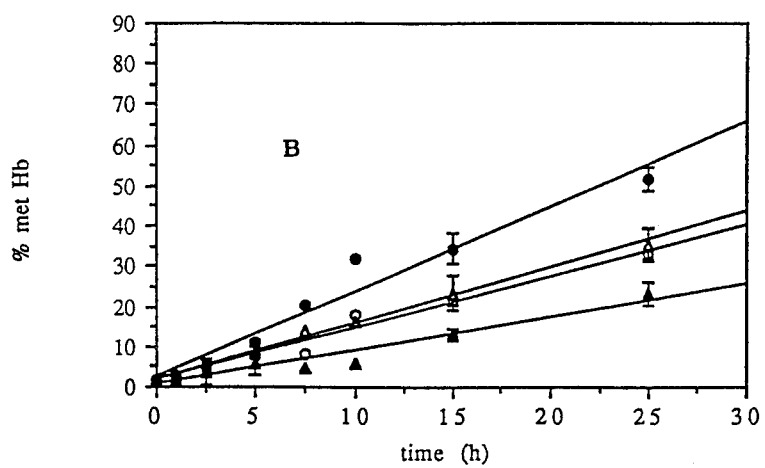
DISCUSSION

The autoxidation of hemoglobin is known to generate superoxide radical, O_2^- [12]. H_2O_2 is a strong oxidizing agent and will oxidize Hb-Fe(II) to Hb-Fe(III). The by-products of this reaction are OH$^-$ and HO$^·$ [13]. The hydroxyl radical is also able to oxidize Hb-Fe(II) to Hb-Fe(III) [3]. If O_2^- and H_2O_2 are responsible for autoxidation, then the break down of these species would retard the autoxidation, as reported here (Table I).

TABLE I

Autoxidation of hemoglobins in the presence or absence of catalase and SOD. The concentrations of hemoglobin was 2.22 mM per heme, and those of catalase and SOD were 5.77 μM tetramers and 23.1 μM dimers, respectively.

Hemoglobins plus enzymes	k_{app}x100 (h^{-1})	$t_{1/2}$ (h)	% In	% In per μM enzyme
Hb A	2.20±0.300	31.5	NA	NA
Hb A+CAT	0.785±0.500	88.3	64	11.1
Hb A+SOD	1.16±0.400	59.7	47	2.0
Hb A+CAT+SOD	0.399±0.160	173.7	82	NA
β82XLHb A	2.69±0.150	25.8	NA	NA
β82XLHb A+CAT	1.38±0.240	50.2	49	8.5
β82XLHb A+SOD	1.52±0.140	45.5	43	1.9
β82XLHb A +CAT+SOD	0.670±0.040	103.4	75	NA
α99XLHB A	3.93±0.680	17.6	NA	NA
α99XLHb A+CAT	1.72±1.44	40.3	56	9.7
α99XLHb A+SOD	2.44±0.266	28.4	38	1.6
α99XLHb A +CAT+SOD	1.29±0.799	53.7	67	NA

% In -total percent of enzyme inhibition.
% In per μM enzyme -total percent of enzyme inhibition per μM of each enzyme.
NA -not applicable

The autoxidation of hemoglobin is bi-phasic [3] and this appears to be the same for crosslinked hemoglobins in the presence of catalase and SOD. In the first phase the methemoglobin rises linearly with respect to time until it reaches 20 h. Beyond 20 h the autoxidation reaches a plateau [3]. This curve shape indicates that the autoxidation involves a multi-step mechanism. The first phase of autoxidation is linear and could be interpreted as a pseudo-first order rate, which is dependent upon the concentration of hemoglobin.

The apparent rate constant (k_{app}) was estimated from the first phase of autoxidation.

The chemical reactions for the multi-step mechanism in the autoxidation of hemoglobin which appear to satisfactorily explain the bi-phasic nature of the curve were proposed by Wallace et al. [12] and modified by Watkins et al. [14]. The oxidation of each Fe(II) to Fe(III) by O_2^- and H_2O_2 was proposed to occur as:

$$Hb\text{-}Fe(II) + O_2 \rightarrow Hb\text{-}Fe(III) + O_2^- \tag{1}$$

$$Hb\text{-}Fe(II) + O_2^- + 2H^+ \rightarrow Hb\text{-}Fe(III) + H_2O_2 \tag{2}$$

$$Hb\text{-}Fe(II) + H_2O_2 \rightarrow Hb\text{-}Fe(III) + OH^- + HO \tag{3}$$

$$Hb\text{-}Fe(II) + HO^{\cdot} \rightarrow Hb\text{-}Fe(III) + OH^- \tag{4}$$
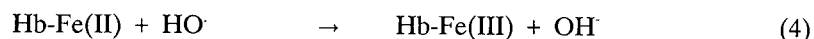
The autoxidation of hemoglobin can be partially inhibited by either catalase and superoxide dismutase (Fig. 1). This is because O_2^-, $HO^{\cdot}$ and H_2O_2 are generated during the autoxidation. Thus, in the autoxidation reactions above SOD would inhibit the progress of reaction 2. By destroying H_2O_2, catalase would protect the hemoglobin from oxidation in reaction 3 and indirectly reaction 4, since the $HO^{\cdot}$ is a by-product of the H_2O_2 oxidation of hemoglobin.

The inhibition observed in both crosslinked and uncrosslinked hemoglobins was 49-64% by catalase and 38-47% by SOD under the conditions used. When both enzymes were used together the inhibition was 67-82%. The $t_{1/2}$ of hemoglobin in the presence of SOD and catalase under these conditions has increased by approximately 4 fold. This effect suggests that hemoglobin or crosslinked hemoglobin to be used as a blood substitute could be protected from autoxidation in long term storage by either or both of these enzymes.

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THERMAL STABILITIES OF HEMOGLOBINS CROSSLINKED
WITH DIFFERENT LENGTH REAGENTS

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ABSTRACT

Bis(3,5-dibromosalicyl) succinate and glutarate were used to crosslink met-, oxy- and deoxyhemoglobins. The added flexibility of these reagents compared to the fumarate analog resulted in a more heterogeneous product but did not greatly affect the maximum thermal stability of the crosslinked hemoglobins.

INTRODUCTION

The interest in crosslinked hemoglobin has increased recently due to its potential as a blood substitute. Human hemoglobin has been stabilized by crosslinking with series of reagents including diaspirins and dialdehyde derivatives of pyridoxal phosphate. The typical example of diaspirins is bis(3,5-dibromosalicyl) fumarate (DBSF) which has been used to crosslink oxyhemoglobin between Lys 82 β_1 and Lys 82 β_2 [1] and deoxyHb between Lys 99 α_1 and Lys 99 α_2 [2]. Thermal denaturations indicated that both α and β crosslinked hemoglobins had a denaturation temperature in 0.9 M guanidine of 57°C compared to 41°C of HbA [3-5]. In this paper, we studied two other derivatives of aspirin: bis(3,5-dibromosalicyl) succinate and glutarate.

719

EXPERIMENTAL PROCEDURES

Materials: The syntheses of bis(3,5-dibromosalicyl) succinate (DBSS) and glutarate (DBSG) are done according to the method of Zaugg et al. [6]. The products were twice recrystallized from a 2:3 hot water-acetone mixture. The melting points were 200°C and 171°C respectively for DBSS and DBSG. The ^{1}H and ^{13}C NMR of these two compounds are consistent with their structures. Hemoglobin was prepared from packed red blood cells according to the method of Dozy, et.al [7].

Crosslinking reactions: The crosslinking reactions of hemoglobin with DBSF, DBSS and DBSG were carried out in 0.01 M MOPS, 1mM NaCN, pH 7.0. In all crosslinking reactions the molar ratio of hemoglobin tetramer to crosslinking reagent was kept at 1:1.1. The oxy hemoglobin concentration was determined spectrophotometrically by using ϵ_{mM} of 13.8 per heme at 540 nm [8]. The percentage of methemoglobin in the sample was determined by the absobances at 542 nm and 576 nm [8]. For the oxy and deoxy crosslinking reactions, there was approximately 8% metHbA in the samples. The crosslinked proteins were isolated by anion exchange chromatography on Sephadex A-50 [7].

Electrophoresis: The amount of crosslinking was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Phast System (Pharmacia). Alkaline agarose electrophoresis was done on a Corning system.

Denaturation experiments: The thermal denaturation experiments were carried out according to White and Olsen [4] and Yang and Olsen [5].

RESULTS

Purification of crosslinked hemoglobin: The reactions of DBSS and DBSG with oxyhemoglobin or of DBSF, DBSS and DBSG with methemoglobin result in two peaks on the ion-exchange chromatography (data not shown); the first one corresponded to uncrosslinked Hb A and the other to crosslinked HbA in all of these reactions. The reaction of DBSS or DBSG with deoxy HbA gave four peaks on the ion-exchange chromatography. The first peak was unmodified HbA. The other three were crosslinked hemoglobins.

Electrophoresis: Figure 1 shows the electrophoretic patterns for the SDS gels of crosslinked oxyHbA and deoxyHbA.   There are three bands in all crosslinked oxyHbA's; two at the position of dimer and the other, monomer. Glutarate crosslinked deoxyHbA had four bands: three, dimer bands and one, monomer.   For peak 2 from succinate crosslinked deoxyHbA , there were two bands at the position of dimer.   These results demonstrate that crosslinking produced a dimeric species with M_r 32,000 and that there are two different crosslinked species for oxyHbA and three for deoxyHbA.   For all reactions with metHbA, the first peak had only a monomer band, but the second had both monomer and dimer bands on SDS-PAGE.   When it was present, the total percent of dimer is close to 50% in all cases.

Denaturation results: Figure 2 shows the effect of the succinate and glutarate crosslinks on the thermal stability of hemoglobin.   The denaturation temperature was determined by the minimum first derivative of absorbance with respect to temperature.   Table I summarizes the T_m values obtained for these species.   For all samples crosslinked under met or deoxy conditions, the T_m's of the first peak were the same as that for uncrosslinked HbA (data not shown).   The larger T_m's of these two crosslinked hemoglobins were approximately 57°C.

DISCUSSION

The SDS-PAGE results for the purified crosslinked HbA's showed three distinct bands after oxy or met crosslinking and four bands after deoxy crosslinking, corresponding to the monomer and the crosslinked dimers. These data indicate that the crosslinking was between subunits.   The additional flexibility of succinate and glutarate over fumarate allow reactions at more sites.   This is demonstrated by the multiple bands in the dimer regions of the SDS gels and by multiple peaks on anion exchange chromatography.

The introduction of the succinate or glutarate crosslink between the subunits increased the T_m by as much as 15°C from that of uncrosslinked methemoglobin, which is similar to that of the fumarate crosslinked protein [3-5]. There are two transition temperatures for some deoxy crosslinked Hb
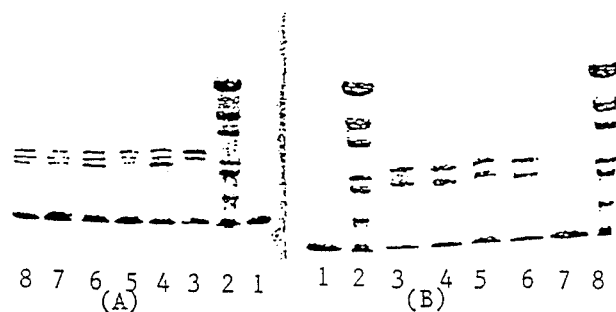
FIGURE 1 SDS gel electrophoresis of crosslinked hemoglobins: (A) Samples crosslinked under deoxy conditions. The lanes are (1) HbA, (2) standard proteins, (3) peak 2 of DBSSXL deoxyHbA, (4) peak 3 of DBSSXL deoxyHbA, (5) peak 4 of DBSSXL deoxyHbA, (6) peak 2 of DBSGXL deoxyHbA, (7) peak 3 of DBSGXL deoxyHbA and (8) peak 2 of DBSGXL deoxyHbA. (B) Samples crosslinked under oxy conditions. The lanes are (1,7) HbA, (2,8) standard proteins, (3,6) DBSSXL oxyHbA and (4,5) DBSGXL oxyHbA. The standard proteins were α-lactalbumin (M_r 14,200), trypsin inhibitor (M_r 20,100), trypsinogen (M_r 24,000), carbonic anhydrase (M_r 29,000), glyceraldehde-3-phosphate dehydrogenase (M_r 36,000), egg albulmin (M_r 45,000) and bovine serum albumin (M_r 66,000).
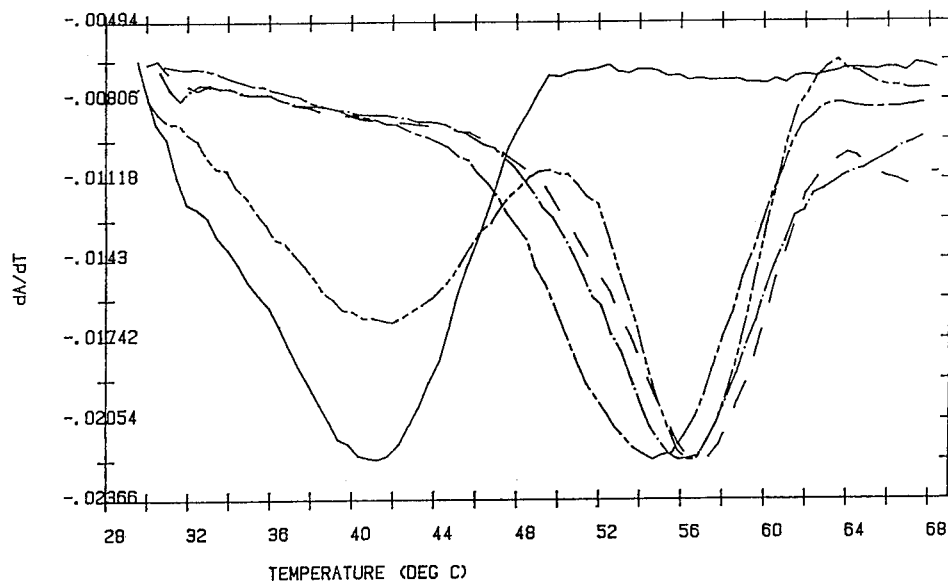
FIGURE 2 Denaturation of crosslinked hemoglobins. The samples were HbA (——), DBSSXL oxyHbA (— —), DBSGXL oxyXLHbA (— · —), peak 2 of DBSSXL deoxyXLHbA (— — —) and peak 2 of DBSGXL deoxyXLHbA (— ·· —).

TABLE I

Denaturation Temperatures of Crosslinked Hemoglobins

Sample	peak #[*]	XL reagent	$T_m(^{\circ}C)^{+}$
HbA		--	41.9±0.9
oxyHbA	2	DBSS	57.4±0.1
oxyHbA	2	DBSG	57.3±0.4
metHbA	2	DBSF	61.0
metHbA	2	DBSS	58.3
metHbA	2	DBSG	60.0
deoxyHbA	2	DBSS	56.0±0.1
deoxyHbA	3	DBSS	56.2±1.0
deoxyHbA	4	DBSS	42.0±1.0, 56.6±0.7
deoxyHbA	2	DBSG	41.7±0.5, 57.0±0.6
deoxyHbA	3	DBSG	39.0±1.0, 54.6±1.3
deoxyHbA	4	DBSG	40.5, 54.7
HbA (oxy or deoxy)		DBSF	57±1.1[#]

[*]Where peaks 2, 3 and 4 are in the order eluted from A-50 column.

[+]The denaturation temperature was determined using data taken at 406 nm.

[#]See references 3 - 5.

species (Table I). The lower one was the same as the T_m for uncrosslinked HbA. Since alkaline agarose electrophoresis did not show any contamination of these sample with uncrosslinked HbA, this means that there are crosslink-ed species with lower thermal stability. It is conceivable that one of the transitions in these samples could be due to the crosslinking of metHbA present in the reaction mixture. However, crosslinked metHbA cannot explain the transition at a lower temperature because its T_m is 58 - 61 °C, depending on the crosslinker used (Table I). The major transition of the peak 4 samples from DBSS or DBSG deoxy crosslinked HbA's were at lower temperatures (40 - 42 °C), although these peaks are chromatographically the

most different from unmodified HbA and are crosslinked to the same degree as peaks 2 and 3 (Figure 1). Multiple T_m's are observed more frequently with DBSG, which is longer and more flexible than DBSS (Table I). All these results indicate that the more flexible and slightly longer crosslinking reagents generate multiple crosslinked HbA's under deoxy reaction conditions, all of which do not have the high stability of the fumarate crosslinked protein.

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INFLUENCE OF THE POLYMERIZATION STEP ALONE ON OXYGEN AFFINITY
AND COOPERATIVITY DURING PRODUCTION OF HYPERPOLYMERS FROM
NATIVE HEMOGLOBINS WITH CROSSLINKERS

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ABSTRACT

The aim of this study was to find out how the polymerization per
se changes oxygen affinity (P50) and cooperativity (n50) of var-
ious soluble huge hyperpolymers prepared from native hemoglobins
by crosslinking. Increase of cooperativity would be expected
considering natural hemoglobin networks. Those hyperpolymers with
molecular weights of some 10^6 g/mol are candidates for artificial
oxygen-carrying blood additives rather than volume substitutes.
Human and bovine hemoglobin reacted with several crosslinkers
(2,5-diisothiocyanatobenzenesulfonate (DIBS); 4,4'-diisothiocyana-
tostilbene-2,2'-disulfonate (DIDS); 1,3-butadiene diepoxide
(BUDE); glutaraldehyde (GDA)) in concentrated (case 1) and diluted
(case 2) hemoglobin solutions. With high concentration hyperpoly-
mer and with low concentration only monomer products were ob-
tained. P50 and n50 of the products were determined at pH = 7.4,
PCO_2 = 40 mmHg, temp. = 37 °C. The difference of properties in
both cases are regarded as the influence of polymerization per se.
Considering this difference we found with almost all combinations
of hemoglobin and crosslinker an increase of O_2 affinity, with
DIBS and DIDS cooperativity was not changed and with BUDE and GDA
it was decreased. As compared with native hemoglobin loss of co-
operativity is considerable in any combination and condition, but
comparing human and bovine hemoglobin the first seems to maintain
better cooperativity. In contrast bovine hemoglobin as compared
with human hemoglobin maintains better or even decreases its O_2

affinity upon reaction with the crosslinkers forming both, monomer
and hyperpolymer products, especially in the deoxy state. DIBS and
DIDS react very similarly. As a general conclusion only deoxy
state reactions led to appropriate products regarding an artifi-
cial oxygen carrier. A differentiated analysis of some samples
clearly indicates crosslinking with increased homotropic coopera-
tivity on going from monomer to hyperpolymer reaction products.

INTRODUCTION

Hyponcotic solutions of hyperpolymer hemoglobins -instead of
isoncotic- may serve as an artificial oxygen carrier like a blood
additive to be applied in case of chronic oxygen deficit of tis-
sues. This new concept amplifies the range of application of
artificial oxygen carriers substantially (1). In 1987 we succeeded
in preparation of soluble huge hemoglobin (Hb) polymers - hyper-
polymers, molecular net works (2) -, and wed were able to demon-
strate in the rat, that those hyperpolymers, having a sufficient-
ly low viscosity, transport oxygen up to 70% of the whole O_2
uptake of the animals (3). The efficacy of modified hemoglobins is
decisively determined by the oxygen binding properties: e.g.
affinity assessed as oxygen partial pressure at half saturation
(P50) and homotropic cooperativity assessed as HILL's index (n or
n50). Chemical modifications of hemoglobin change both. On the
other hand, a high degree of association renders possible high n-
values, as is shown in the natural hyperpolymer erythrocruorin of
the earthworm comprising about 200 Fe-atoms and reaching n-values
of 12 (4). In regard to our new concept we were interested to know
how the polymerization step per se influences the oxygen binding
properties. For this purpose human and bovine hemoglobin were
allowed to react with 2,5-diisothiocyanatobenzenesulfonate (DIBS),
4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), 1,3-butadi-
ene diepoxide (BUDE), and glutaraldehyde (GDA) in either highly
concentrated and diluted Hb solution. The crosslinkers react with
amino groups of the hemoglobins. In the first case hyperpolymeric,
in the second case only monomer hemoglobin is obtained. The dif-
ference of the oxygen binding properties of reaction product
yielded in concentrated and diluted solution is regarded as the
influence of the polymerization step per se. Berbers et al. could
not find a dependency of P50 with degree of polymerization cross-
linking pyridoxylated human Hb with GDA (5).

MATERIALS AND METHODS

Human blood was obtained from the cubital vein of young healthy
donors, bovine blood from a slaughter-house. General procedure
(see also table I): Plasma was removed by washing with 150 mM
$NaHCO_3$ or BiKu-solution (125 mM NaCl, 4.5 mM KCl, 20 mM $NaHCO_3$;
1 M = 1 mol/l). Hemolysis was done by cryolysis. The solutions had
a Hb content of about 300 g/l and a pH of 8.0 and 8.4, respective-
ly. For monomer preparation the cryolysate was diluted to about
10 g/l with BiKu-solution, for preparation of hyperpolymers the
solution was undiluted. Deoxygenation was done by overflow of the
stirred solutions with nitrogen. The volumes of the reaction
mixtures were between 300 μl and several ml. The reaction tempera-
ture was 4 °C, and the time about 20 h. The crosslinkers were
added in ten-fold molar excess related to Hb4. In the case of
reaction at high concentration, the amount of crosslinker and
reaction time were adapted to get the highest yield of soluble
reaction products (about 90%). DIBS and DIDS were solved in di-
methylsulfoxide before addition. The reactions were terminated by
addition of lysine in 30-fold molar excess related to the cross-
linker. Separation of hyperpolymers was done with ultrafiltration
(cut off 300 kD). The overall yield was about 40%. Met-Hb con-
tent was lower than 10%. Gelchromatography (GC) of the hyperpoly-
mers was done with Sephacryl HR (200/400/500) purchased from
Pharmacia, Freiburg/D (6). The oxygen binding properties of the
samples were determined with a spectrometric thin layer method
(7): the layers were equilibrated with various gases via a teflon
membrane. Milieu: BiKu-solution at 37 °C, pH = 7.4 and PCO_2: 40
mmHg in a 20 g/l Hb concentration. The error of the method is
about 10% in P50.

RESULTS

Gelpermeation chromatography reveals a broad distribution of the
hyperpolymer hemoglobins with some 10^6 g/mol weight average molec-
ular weights as evidenced by light scattering (8); in diluted
solutions no polymers were found, which was shown by GC on Seph-
acryl 200 HR.
Table I presents all results of oxygen binding measurements in
cumulo.

Table I: Oxygen binding properties of chemically modified human
             and bovine hemoglobin with different crosslinkers

cross-linker	blood	degree of polymerization (hyperpolymer/monomer)	O$_2$-status (oxy/deoxy)	P50 (mmHg)	n50[l]	remarks
—	human	—	—	18	2.6	native Hb
	bovine	—	—	25	2.5	
DIBS	human	hyperpolymer	oxy	5	1.4	a
		hyperpolymer	deoxy	10	1.5	a
		monomer	oxy	6	1.5	b
		monomer	deoxy	21	1.6	b
	bovine	hyperpolymer	oxy	6	1.2	a
		hyperpolymer	deoxy	25	1.3	a
		monomer	oxy	14	1.0	a
		monomer	deoxy	77	1.1	a
DIDS	human	hyperpolymer	oxy	3	1.1	a
		hyperpolymer	deoxy	14	1.4	a
		monomer	oxy	12[i] - 15[j]	2.1[i] - 1.8[j]	b,k
		monomer	deoxy	28	1.5	b
	bovine	hyperpolymer	oxy	3	1.1	a
		hyperpolymer	deoxy	31	1.3	a
		monomer	oxy	4[i] - 23[j]	1.3[i] - 1.1[j]	b,k
		monomer	deoxy	59	1.4	b
BUDE	bovine	hyperpolymer	oxy	2	1.0	a
		hyperpolymer	deoxy	25	1.0	b,c,d
		monomer	oxy	12	1.6	b
		monomer	deoxy	22	2.0	b
GDA	human	hyperpolymer	oxy	8	1.3	b,c,e,f,g,h
		hyperpolymer	deoxy	13	1.5	b,c,e,f,g,h
		monomer	oxy	13	2.2	b,c,h

a: blood cell washing with 150 mM NaHCO$_3$; b: blood cell washing with BiKu-solution; c: reaction at 22 °C; d: desoxygenation by absorption of O$_2$ with Na-dithionite in closed vessel; e: crosslinking within intact erythrocytes; f: no separation of hyperpolymers by ultrafiltration; g: reaction not stopped with lysine; h: reaction product not reduced; i(j): values at the beginning (end) of repeated measurements with one layer; k: after reaction bright yellow precipitate; l: average values between S ca. 0.3 and S ca. 0.8

In the case of DIDS/monomer/oxy/human and bovine we found P50 to increase and n50 to decrease during repeated measurements. Such an instability was also observed by Kavanaugh et al. with human Hb and DIDS (10). In all cases, except BUDE/bovine/deoxy, the O_2 affinity increases 2-fold to 5-fold turning from monomer to hyper-polymer. In contrast, cooperativities do not change with DIBS and DIDS, but they decrease with BUDE and GDA. P50 and n50 of native human Hb at these conditions are 18 mmHg and 2.6 and of bovine Hb 25 mmHg and 2.5, respectively. As is shown (table I) all chemical modifications reduce these cooperativities greatly but not P50: Both DIBS and DIDS increase P50 in bovine and human deoxy hyper-polymers and in bovine deoxy monomers demonstrating the similar-ity of the two crosslinkers in their reaction. In each case, changing from the deoxy to the oxy state increases O_2 affinity, but not cooperativity, which is constant in all cases, except with BUDE/bovine/monomer, where it decreases. Comparison of both hemoglobins shows that human Hb preserves better cooperativity whereas bovine Hb gives lower O_2 affinities. Regarding and compa-ring the data of table I it is hard to see clear general rules (without exceptions) as to the effect of the crosslinkers on P50 and n50 values.
Hill's index is not a constant but depends in a characteristic manner on O_2 saturation (cooperativity function). This demon-strates figure 1 for human whole blood, according to data from Severinghaus (19) and for some of the modified hemoglobins as indicated in the legend.
Fig. 1 shows, that in case of bovine Hb with DIBS in deoxy state the polymerisation increases cooperativity at high O_2 saturation. It shows further, that human Hb hyperpolymer crosslinked with GDA reaches at high O_2 saturation the cooperativity of whole blood, but at low O_2 saturation the cooperativity is, by far, lower.

DISCUSSION

The reaction of the hemoglobins with the crosslinkers may result in a heterogeneity of monomer products - especially with regard to intratetramer crosslinking -, which were not separated, so oxygen binding data presented here may be average values. DIBS, DIDS, BUDE and GDA were also used by other investigators with human Hb (9,10,11,12, respectively). As to DIBS and DIDS our findings confirm the hypothesis of Marinov and Rusieva (13), which states that small electron donor molecules decrease O_2 affinity. A gener-
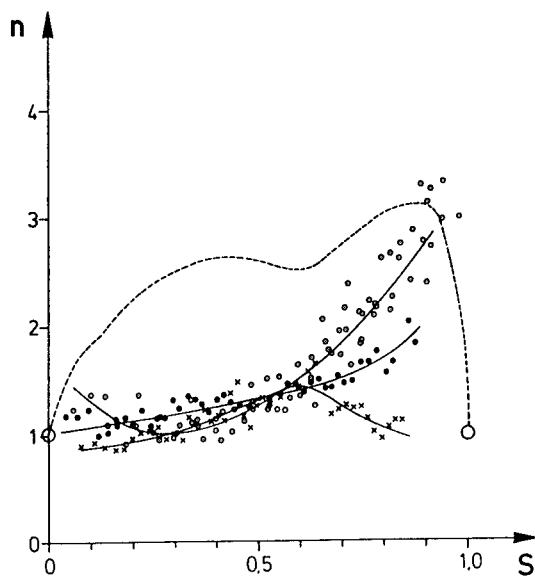
<u>Fig. 1</u>: cooperativity functions of normal human blood (---) and
various modified hemoglobins; S: O_2 saturation, n: HILL's
index; temp.: 37 °C, PCO_2 = 40 mmHg, pH = 7.4, in BiKu-
solution;   ● ● ● bovine/DIBS/deoxy/hyperpolymer; xxx bo-
vine/DIBS/deoxy/monomer;ooo human/GDA/deoxy/hyperpolymer

al conclusion drawn from the results presented here is that, if at
all, only reactions in deoxy state lead to usable products as to
an artificial oxygen carrier.

Often characterization of cooperativity only by the average quan-
tity n50 is not sufficient. It is better to use the whole coopera-
tivity function with its characteristic form: so-called fine
structure (14,15). Indication for this comes also from calculation
of residuals of measured curves and theoretical functions
(16,17,18). High n-value at low O_2 saturation facilitate oxygen
release from the capillaries to tissues, high n-values at high O_2-
saturation facilitate oxygen uptake by the lung.

The selected example of a differentiated analyses of cooperativi-
ty, given in fig. 1, shows, that indeed it is possible to increase
cooperativity of hemoglobin by polymerisation, which is expected
regarding natural hemoglobin networks. This increase of coopera-

tivity could not have been shown by simply using n50 as a cooperativity parameter.
It is very desirable to design modified hemoglobins with cooperativity function resembling that of human blood.

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CHIMERIC HEMOGLOBIN SUBUNITS: FUNCTIONAL PROPERTIES OF A RECOMBINANT β/α HEMOGLOBIN

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ABSTRACT. Our goal was to design a single hemoglobin subunit able to assemble into a stable tetrameric structure with cooperative O_2 binding and low oxygen affinity. We have synthesized in *E. coli* a chimeric β/α globin subunit composed of the first 73 residues of the β chain and the last 73 residues of the α chain. Molecular building indicated that this construction could result in Hb homotetramers possessing the $\alpha_1\beta_2$ interface, responsible for the heme-heme interaction in Hb heterotetramers. The results show that the chimeric subunits assemble into tetramers which bind oxygen reversibly without cooperativity but with an oxygen affinity slightly lower than observed for human Hb. The strong effector RSR 4 lowers the oxygen affinity. Kinetics of CO recombination in the presence of RSR 4 reveal a biphasic bimolecular rebinding. Functional studies suggest that the quaternary structure of the oligomer is intermediary between R- and T-state.

INTRODUCTION

The development of an artificial oxygen carrier based on human hemoglobin (Hb) requires at least two modifications of the Hb molecule: i) reduction of the oxygen affinity in the absence of 2,3-diphosphoglycerate (DPG); ii) stabilization of the

tetrameric structure of Hb in solutions. The recent advances in genetic engineering have facilitated the design and expression of mutant Hb with a desired oxygen affinity [1, 2]. Site-directed mutagenesis has also been successful in achieving human Hb $\alpha_2\beta_2$ with a glycine linking the 2 α chains, to prevent dissociation into dimers [3]. Our purpose has been to design a single hemoglobin subunit able to assemble into a stable tetrameric structure with low oxygen affinity. Isolated β Hb chains assemble into homotetramers exhibiting a very high O_2 affinity. Introduction of several different mutations at the β/β interface was unsucessful in lowering the oxygen affinity, and/or creating heme-heme interaction. Another approach was to construct a chimeric Hb subunit able to assemble into tetramers which may bind oxygen cooperatively and with a low oxygen affinity. We have produced a chimeric protein in which the 73 N-terminal residues of the β globin chain are fused to the last 73 residues of α (Fig. 1). This construction should reconstitute the $\alpha_1\beta_2$ interface responsible for the heme-heme interaction in the heterotetramer $\alpha_2\beta_2$.

MATERIALS AND METHODS

Dimer-tetramer equilibrium: The assembly of the chimeric subunits into oligomers was verified by crosslinking with glutaraldehyde, followed by SDS-polyacrylamide gel analysis [4].

Expression and purification of the protein: The chimeric β/α globin subunit was produced as a fusion protein in *Escherichia coli*, using the expression vector pATPr cIIFX β73-69α. The cIIFX-β73-69α coding sequence was constructed starting from pATPr cIIFX-β [5] containing the β-globin cDNA, and αpJW101 (provided by Dr B. Forget) containing the α1-globin cDNA, using standard methods [6, 7]. After extraction and purification, the fusion protein was cleaved by digestion with bovine coagulation factor Xa. The β/α subunits were folded in the presence of cyanohemin, and the tetramer purified by chromatography [5, 8].

Spectrophotometric studies: Static visible spectrophotometric measurements were obtained for the Hb solutions in 0.1 M NaCl, 50 mM Tris-HCl buffer pH 7 at 25°C with a Cary 219.

Functional studies: Oxygen equilibrium curves were recorded with a continuous method [9] using the Hemox Analyzer system (TCS, Huntington Valley, PA, USA). Kinetics of CO recombination were recorded at 436 nm, after
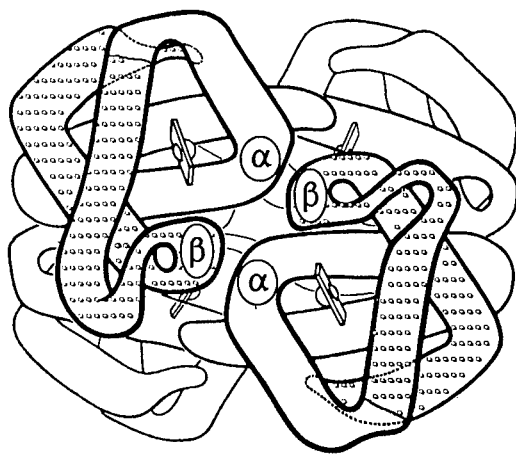
FIGURE 1. Schematic representation of the association of chimeric hemoglobin subunits. This construction should reconstitute the $\alpha_1\beta_2$ interface responsible, in the heterotetramer Hb A, for the heme-heme interaction.

flash photolysis using a 10 ns pulse providing 160 mJ at 532 nm (Quantel YAG laser) [10].

RESULTS

The static visible spectra obtained with the CO form of the reconstituted chimeric Hb was similar to that of COHb A. In the oxygenated form the α band peak was increased compared to the β band peak, suggesting the presence of a hemichrome [11]. Isoelectric focusing revealed a single band migrating between Hb A and the homotetramer β_4.

The chimeric subunits assemble into homotetramers as shown by glutaraldehyde linkage. The electrophoresis pattern on SDS polyacryamide gel electrophoresis revealed the presence of two major components, similar to those obtained for normal human Hb A, corresponding to tetrameric and dimeric hemoglobin chains.

The chimeric homotetramers bind oxygen reversibly with low cooperativity. The P_{50} was slighty higher than that of human Hb A, and 30 times increased compared to that of the homotetramer β_4. RSR 4, a potent effector interacting
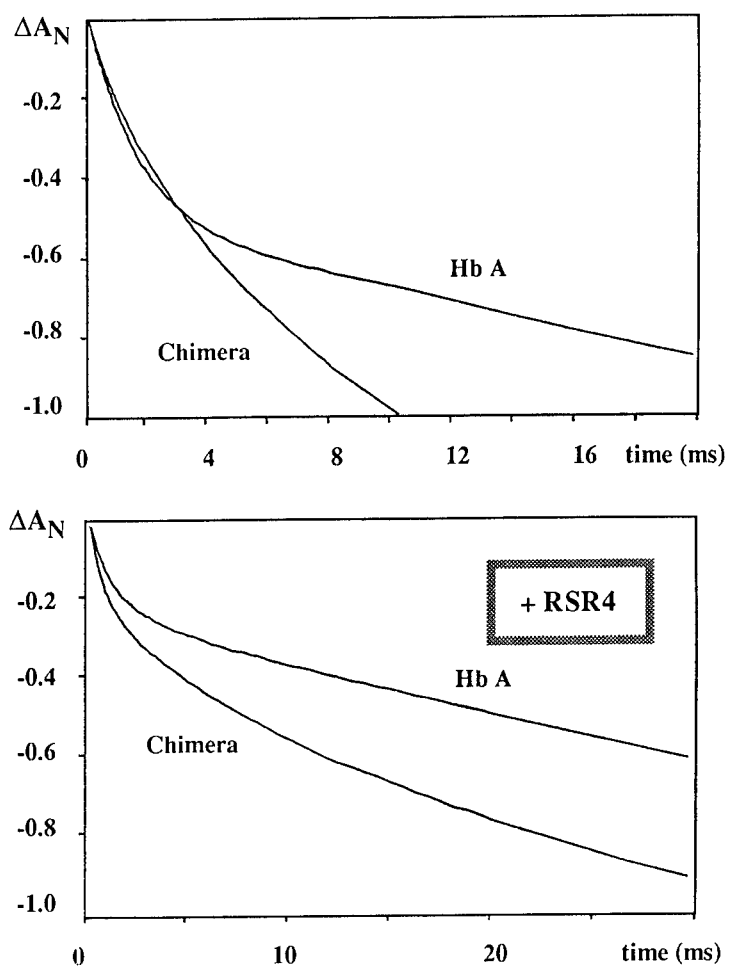
FIGURE 2. Recombination of CO to the chimeric hemoglobin and to Hb A, after photodissociation of the ligand, in the presence and in the absence of 0.3 mM RSR 4. The kinetic curves are normalized and presented on a log-linear plot. Upon addition of the effector RSR 4, both Hb A and the chimera show an increase in the slow phase, characteristic of deoxy (T-state) Hb. Experimental conditions were: 50% photodissociation, 100 mM NaCl, 50 mM bisTris at pH 7.2, 25°C, 0.1 atm CO, 80 mM heme.

predominantly with α chains in tetrameric Hb A [12] lowered markedly the affinity.

In our standard experimental conditions (50% photodissociation, 0.1 atm CO, pH 7.0) the CO recombination kinetics for Hb A are biphasic: the initial fast phase corresponds to the recombination of CO to the high affinity R-state, while the slow phase is ascribed to the low affinity T-state. The relative amplitudes of these two phases depends on the fraction photodissociation and on external effectors, indicating the presence of an allosteric mechanism. In Hb A the two rates are decreased upon addition of strong allosteric effectors such as inositol hexakisphosphate or RSR 4 [12]. In the absence of strong effector, the kinetics of CO rebinding to the chimera was almost monophasic; the rate of CO rebinding was about two-fold lower than that measured for Hb A in the R-state (Fig. 2). Thus, both equilibrium and kinetic data indicate that the chimera is in an intermediary structure, between T- and R state. Addition of saturating amount of RSR 4 to the chimeric Hb solution resulted in biphasic kinetics, the slower "T" rate being faster than the corresponding rate in Hb A (Fig. 2). This observation confirmed the equilibrium studies indicating that the chimeric Hb is, at least partially, in a tetrameric form exhibiting effector dependent properties.

DISCUSSION

We have constructed a chimeric Hb subunit composed of the 73 N-terminal residues of the β globin chain and the 73 C- terminal residues of the α chain (Fig. 1). This contruction was selected in the view of obtaining a single Hb subunit able to assemble into a stable tetrameric structure reconstituting the $\alpha_1\beta_2$ interface. Functional studies show that the chimeric subunits bind oxygen reversibly with an affinity much lower than that of the homotetramer β_4. However, there is apparently a slight perturbation of the $\alpha_1\beta_2$ interface which does not allow the chimera to bind oxygen cooperatively. The low cooperativity and the low oxygen affinity of the chimeric homotetramer may be explained either by the existence of a new state (R' or T') with intermediate affinity, or by a mixture of R- and T structures insensitive to the number of ligands bound.

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FUNCTIONAL PROPERTIES OF β(NA1)VAL-DELETED,(NA2)HIS→MET HEMOGLOBIN SYNTHESIZED IN *ESCHERICHIA COLI*.

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ABSTRACT

Bovine Hb (hemoglobin) has a low oxygen affinity in the absence of chloride ions and DPG. Because of the increasing interest of this Hb as a potential blood substitute we have engineered a human Hb mutant with the aim of mimicking the functional properties of bovine Hb. This was achieved by deleting residue βNA1 Val and substituting a methionine for histidine at the βNA2 position as previously suggested by Perutz and Imai in 1980. Our results show that the artificial mutant exhibits some of the characteristics of bovine Hb but does not show the low oxygen affinity which is measured in bovine blood.

INTRODUCTION

On the basis of their blood oxygen affinity, Bunn [1] reported that mammalian hemoglobins could be divided into two groups: those with a high intrinsic affinity which is lowered in the red cells by 2,3-diphosphoglycerate (DPG) and those with low oxygen affinity despite of the absence of DPG in their erythrocytes. The two main prototypes for these Hbs are human Hb and bovine Hb respectively. Low oxygen affinity Hbs were also discovered in several species such as ovidae, felidae, and a primate, the lemur. Perutz and Imai [2] noted that all mammalian Hbs with high intrinsic oxygen affinity have hydrophilic residues in position βNA2 while those with low oxygen affinity have hydrophobic ones, either

739

because residue βNA2 is deleted and replaced by an N-terminal Met, as in bovine, or because it is replaced by Phe or Leu as in cat and lemur Hbs.

They suggested that the hydrophobic side chains of these residues "adhere to the hydrophobic interior of the β chains and pull the two helices A towards the center of the molecule so that they become locked more tightly to neighbouring segments of the polypeptide chain", thus mimicking the effect of DPG on human deoxyhemoglobin described by Arnone [3]. This hypothesis can now be put to the test since site directed mutagenesis permits one to change any residue in the protein. We have therefore engineered the deletion of the βNA1 Val and the substitution of βNA2 Met for His and have studied the functional properties of this new artificial mutant. If minor changes were sufficient to lower the oxygen affinity of human Hb without requiring DPG and without increasing the susceptibility of Hb A to autooxidation, this new mutant would be a good candidate for a blood hemoglobin-based substitute.

METHODS

Human and bovine blood (Holstein breed cattle) were collected on heparin. The red cells were washed in isotonic buffer, lysed in bi-distilled water and the hemolysate was processed as described in [4]. The purity of the Hb samples was checked by isoelectric focusing. Oxygen equilibrium curves were recorded with the Hemox Analyzer (TCS, Huntington Valley, PA, USA) as described in detail elsewhere [4]. Typical samples were 60 μM heme, 100 mM sodium chloride in 50 mM bisTris buffer, pH 7.2 at 25°C. Experiments in the absence of chloride were performed in 10 mM Hepes buffer at the desired pH. No difference was observed in the oxygen binding values whether 10 or 50 mM Hepes buffer was used. The data were analysed according to the formalism of the two-state allosteric model [5].

Kinetics of CO recombination were recorded after flash photolysis by a 10 ns pulse providing 160 mJ at 532 nm (Quantel YAG laser). Samples were 0.1 mM heme in 1-mm cuvettes, with observation at 436 nm [6]. Curve fitting procedures for the calculation of the CO recombination rates were performed as described in [6]. Spectrophotometric (SLM-Aminco DW2000) and fluorimetric studies (SLM 8000) were carried out as routinely used in this laboratory.

The mutated β-globin subunit was produced as a fusion protein in *E. coli* using

the expression vector pAT PrcII FXβ [7]. After extraction and purification, the fusion protein was cleaved by digestion with bovine coagulation factor Xa. The β subunits were folded in the presence of cyanohemin and the partner α subunit, prepared from native Hb A, to form the tetrameric Hb. The structure of the mutated chain was checked by reverse-phase high performance liquid chromatography of the tryptic digest and amino-acid analysis of the mutated peptide.

RESULTS

I- Functional properties of bovine Hb : homotropic effects

Oxygen equilibrium binding experiments of bovine Hb in *chloride free medium* revealed an almost 3 fold decrease of the intrinsic oxygen affinity of bovine Hb relative to HbA (Table I).

In the absence of chloride, bovine Hb exhibits the same P_{50} value (5.1 torr) as Hb A in the presence of 100 mM chloride (5.1 torr); in the presence of 100 mM chloride, the P_{50} for bovine Hb is similar (14.5 torr) to that of human Hb A in the presence of 1 mM DPG (14.1 torr) (Table I). These results agree with numerous data from different laboratories which have demonstated that bovine Hb exhibits a low intrinsic oxygen affinity [2, 8]. They are not in agreement with recent statements by Fronticelli [9] that the affinity for oxygen of bovine and human Hbs were alike in anion-free media. We are not aware of any difference in the amino acid sequence between the β chains of the Holstein breed cattle used in this laboratory and those of Hereford cattle used by the Baltimore team. Kinetics of CO recombination after flash photolysis confirmed the equilibrium data.

Another characteristic of the oxygen binding property of bovine Hb is its large cooperativity value (n_{50}) being constantly higher than for Hb A solutions, even in the absence of chloride or in the presence of the strong effector IHP.

The titration curve with chloride anions for bovine Hb is shifted to higher P_{50} values relative to Hb A but remains parallel to it, indicating an identical oxygen-linked chloride binding for the two Hbs (Figure 1). At variance with Fronticelli [9] we were unable to find evidence for extra chloride binding sites for bovine Hb.

Bovine Hb reacts weakly with DPG in chloride free media and not at all in the presence of 100 mM chloride. Table 2 also indicates a slight decrease of the value of the alkaline Bohr effect for bovine Hb relative to that measured for Hb A.

BAUDIN ET AL.

Table I : Oxygenation parameters for Hb A,
human rec-Hb β1Val deleted-β2Met (E. coli) and bovine Hb

Exp.Cond.	Hb A**			bovine Hb**			rec-Hbβ1del.β2Met		
	P_{50}	n_{50}	K_T	P_{50}	n_{50}	K_T	P_{50}	n_{50}	K_T
Cl=0*	1.8	2.1	6.3	5.1	2.8	33.0	2.1	2.0	4.9
Cl 0.1M	5.1	2.7	25.3	14.5	3.0	81.8	6.0	2.0	13.3
Cl 0.1M +DPG 1mM	14.1	2.7	53.3	17.6	2.9	83.3	9.3	2.2	20.6

Other conditions : pH 7.2, bisTris 0.05 M, 25°C

*Hepes 0.01 M.
** native hemoglobins
P_{50} and K_T values are in mm Hg.
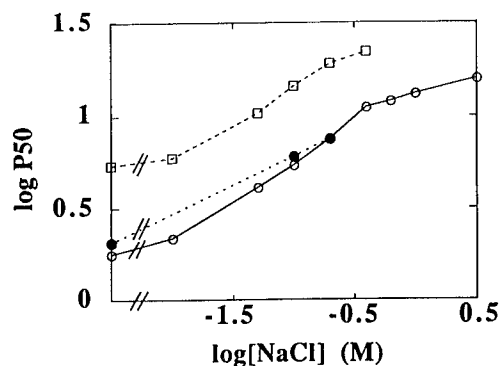
FIGURE 1 : Titration curves of Hbβ1del.β2Met (●), bovine Hb (❑), and
Hb A (O) solutions with chloride.
Other conditions : pH 7.2, bisTris 0.05M with chloride or
Hepes 10mM without chloride, 25°C.

Table II : Heterotropic effects for Hb A,
human rec-Hb β1Val deleted- β2Met (E. coli) and bovine Hb

	Hb A	bovine Hb	rec-Hbβ1del.β2Met
DPG Effect* :			
Cl=0	0.86	0.55	0.71
Cl 0.1M	0.44	0.08	0.19
Bohr Effect* :			
Cl 0.1M	-0.55	-0.40	-0.48

*$(\Delta logP_{50} \pm 1mM$ DPG)
*$(\Delta logP_{50}/\Delta pH$:6.5-7.5)

II- Site directed mutagenesis at the βN-terminal region of Hb

In the absence or presence of chloride anions the oxygen affinity of the β(NA1)Val–deleted,(NA2)His→Met mutant Hb is 20% lower than that of Hb A. This mutant has lower n_{50} values than native Hb A or bovine Hb (Table I). Table II shows a slightly decreased DPG effect in chloride free buffer for the mutant relative to Hb A and a large inhibition of the DPG effect in the presence of chloride however to a lesser extent than in bovine Hb. Note also that the value of the alkaline Bohr effect for the mutant is smaller than normal and close to that of bovine Hb.

DISCUSSION

Removal of the β-chain N-terminal valine associated with the β2His→Met mutation does not allow one to mimic all the functional properties of bovine Hb especially its homotropic effects (oxygen affinity, co-operativity). However the new artificial mutant Hb exhibits some of the heterotropic effects similar but not identical to those of bovine Hb (weak DPG interaction in the presence of chloride, low Bohr effect). Similar results have also been observed by Fronticelli et al [10] in their studies of the identical artificial β chain mutant.

Certain of the amino acid differences between bovine and human may be responsible for these results. Among these are the β5 (A2) Pro and the

Table III : Bovine and human β globin sequences

site	2,3-DPG site				5	130
	1	2	82	143	A2	H8
	NA1	NA2	EF6	H21		
human Hb	Val	His	Lys	His	Pro	Tyr
bovine Hb	-	Met	Lys	His	Ala	Phe

substitution of β130 (H8) Tyr (Table III) which may be involved in the rigidity of the A helix and may prevent enough mobility for the methionyl residue to lock the entrance of the cavity between the two β chains as DPG does in human Hb A. These additional mutations are currently being studied in our laboratory.

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BISSULFOSUCCINIMIDYL ESTERS OF ALIPHATIC DICARBOXYLIC ACIDS: A NEW CLASS OF 'AFFINITY DIRECTED' $\beta\beta$ CROSSLINKERS OF HbA

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ABSTRACT

The potential of sulfosuccinimidyl ester of suberic acid as an intramolecular crosslinker of HbA, directed to the positive charge rich domain of the protein (DPG pocket) has been investigated. The suberate ester introduced crosslinks between the β-chains and inhibited the dissociation of HbA into $\alpha\beta$ dimers. The facile crosslinking reaction seen with this suberate ester as compared to the absence of crosslinking with the diaspirin derivative of suberic acid suggests the strong 'steering' influence of the sulfosuccinimidyl moiety of the reagent to the targeted site. The application of this crosslinking approach in the preparation of Hb based blood substitutes is discussed.

INTRODUCTION

Aliphatic carbonyl reagents that carry negative charge (carboxyl or phosphate) at their distal end exhibit a preferential reaction at the α-amino group of the β-chain in the reductive alkylation of oxy HbA and oxy HbS [1,2]. In contrast, the corresponding uncharged aldehydes exhibit nearly the same reactivity towards the α-amino groups of both the α and the β-chains [3,4]. The distinct differences in the site selectivity during the alkylation of Hb by uncharged and negatively charged aldehydes is apparently a consequence of the 'steering effect' afforded by the negative charge

of the aldehyde to the DPG binding pocket of the molecule. This region of Hb has a very high density of positive charges with a constellation of eight positive charges, i.e. it is a 'proton rich domain' of HbA. Therefore, we have now reasoned that reagents that carry a negative charge in their leaving groups will also exhibit such a preferential reaction at the $\beta\beta$ interface, i.e. such reagents may be 'affinity directed'. We have now initiated a systematic study to evaluate the potential of some of the commercially available general protein crosslinkers that may act as 'affinity directed' $\beta\beta$ crosslinkers. The initial choice was to investigate bifunctional reagents carrying a negatively charged moiety in their leaving group. Such reagents will not introduce an extrinsic negative charge to the 'proton rich domain' of HbA as a consequence of the crosslinking process. Bis sulfosuccinimidyl esters of aliphatic dicarboxylic acids (Fig 1) have been now evaluated as the potential $\beta\beta$ crosslinkers.

MATERIALS AND METHODS

The sulfosuccinimidyl esters were purchased from Pierce Chemical Company, except for Bis sulfosuccinimidyl sebacate which was synthesized for the first time. The preparation of HbA and the HPLC analysis of the globin chains were carried out as described before [2]. The reaction of oxy HbA with sulfosuccinimidyl esters were carried out in phosphate buffered saline at pH 7.4 and room temperature. Superose-12 gel filtrations were carried out using the FPLC system from Pharmacia.

RESULTS

Selectivity of the reaction of sulfosuccinimidyl acetate to the β-chain of HbA: Before undertaking the crosslinking studies with the bifunctional reagents, the reactivity of sulfosuccinimidyl acetate (a monofunctional reagent) with HbA was investigated, particularly to determine whether a selectivity for the β-chain exists as postulated. A 30 minute reaction of HbA (0.5 mM) with a 5 fold molar excess of the reagent at room temperature, followed by RPHPLC of the reaction product showed that the β-chains are derivatized extensively. On the other hand, the α-chains hardly reacted with the reagent even when the reagent concentration is increased to ten fold molar excess. Thus sulfosuccinimidyl acetate exhibits a high degree of selectivity to modify

the functional groups of the β-chain. This is supportive of our postulation that the sulfosuccinimidyl moiety of the reagent can 'steer' it to the 'proton rich domain' of the protein, namely to the DPG binding pocket.

Crosslinking of HbA by sulfosuccinimidyl suberate: Initial attempts to crosslink HbA with bifunctional sulfosuccinimidyl esters were

Figure 1. Acylating reagents.

carried out with the suberic acid a spacer arm of six carbons between the two carboxyl groups) derivative, as the spacer arm of this bifunctional reagent appears to be reasonably consistent with the disposition of the amino groups within the DPG binding pocket to favor the formation of intramolecular crosslinks, namely inter $\alpha\beta$ dimer crosslinks. RPHPLC map of the globin chains of HbA (0.5 mM) reacted with a 5 fold molar excess of sulfosuccinimidyl suberate for 30 minutes at pH 7.4 and room temperature revealed that like the monofunctional sulfosuccinimidyl acetate, the chemical reaction with the sulfosuccinimidyl suberate is also specific for the β-chain. The α-chain appeared to be refractory for reaction with the suberate. However, the HPLC pattern is quite distinct from that of the sulfosuccinimidyl acetate treated material. In the bifunctional reagent treated sample, new components eluting after the position of the α-globin are present; such components were absent in the sample of HbA reacted with the monofunctional reagent. Presumably, these components represent the crosslinked β-globin.

The sulfosuccinimidyl suberate reacted HbA was subjected to gel filtration on a column of Superose-12 equilibrated and eluted with phosphate buffered saline pH 7.4 in order to establish the molecular mass of the modified protein. Nearly 90% of the modified protein eluted in the region of 64 K with less than 10 % of the material eluting around 128 K region (inter tetrameric crosslinks). Moreover, the suberate

reacted HbA retained essentially the same molecular mass (64 K) even when it was subjected to gel filtration on the Superose 12 column in 50 mM Bis-Tris buffer, pH 7.2 containing 100 mM EDTA and 1 M $MgCl_2$. Under these high salt conditions, unreacted HbA dissociates into $\alpha\beta$ dimers leading to a lowering of its molecular mass [5]. The demonstration that the suberate reacted HbA retains the molecular mass of the intact tetramer (64 K) even under the conditions that favor dissociation of the tetramer establishes that suberate has introduced intratetrameric crosslinks. Since the α-globin has essentially remained underivatized, it is apparent that bis sulfo-succinimidyl suberate is an efficient $\beta\beta$ crosslinker.

Influence of the length of the spacer arm of the bifunctional sulfosuccinimidyl esters on the crosslinking reaction: The propensity of bis sulfosuccinimidyl tartarate and sulfosuccinimidyl sebacate to introduce crosslinks into HbA was also investigated and compared with that of suberate. The tartarate, which has a two carbon alkyl chain between the reactive carboxylates failed to introduce crosslinks. On the other hand, the sebacate, with eight carbon alkyl chain as the spacer arm introduced the crosslink with an efficiency nearly comparable to that of suberate.

DISCUSSION

The studies presented here demonstrate that the sulfosuccinimidyl esters of aliphatic carboxylic acids are steered to a region of HbA that facilitate the acylation of the functional groups of the β-chains. We speculate that the facile sulfo-succinimidyl ester mediated acylation occurs with the amino groups of the DPG binding pocket. This region of Hb represents a 'proton rich domain' of the protein with a constellation of eight positive charges under the physiological conditions. Thus, the 'steering' of the negatively charged chemical modification reagents to this positive charge dense domain of the molecule is not surprising. Four of the positive charges of this region come from the amino groups, the potential sites for the acylation reaction. Therefore, a bifunctional reagent with a molecular geometry at its tagging ends that complements the disposition of the amino groups within the DPG binding pocket and which also incorporates the sulfosuccinimidyl chemistry as a steering force to direct the reagent to the pocket may be expected to generate intramolecular cross-linking. The suberate ester indeed satisfies this requirement, since the spacer arm of

the reagent can span a maximum length of around 11 Angstroms. Based on the crystal structure of HbA, this distance is compatible for the formation of interchain, intratetrameric crosslinks in HbA by bridging the α-amino group of Val of one β-chain with the e-amino group of Lys-82 of another β-chain. However, the spacer arm of suberate may be a little long to bridge the two Lys-82(β) residues and too short to bridge the two Val-1(β) residues of DPG pocket. But, a certain degree of flexibility is expected for the alkyl chains of the reagents.

Introduction of intramolecular crosslinks involving the amino groups of the DPG binding pocket has been achieved through the use of other acylating agents, particularly diaspirins [6] and methyl acetyl phosphates of di and /or tricarboxylic acids [7]. The reaction of diaspirins of aliphatic dicarboxylic acids are particularly relevant to the investigations of the present study. Though the diaspirin of succinic acid introduced $\beta\beta$ crosslink into HbA [8], the diaspirins of adipic, suberic and sebacic acid failed to introduce intramolecular crosslinks with oxy HbA at pH 7.2. It has been suggested by Klotz and his colleagues [8] that one of the factors responsible for the high reactivity of the diaspirins (as compared with the monoaspirins) is that the two rings of the diesters provide strong apolar interactions to facilitate the binding of the regent to the protein. The results of the present study with bis sulfosuccinimidyl esters demonstrate that the leaving group of these bifunctional reagents provide interactions that are unique and distinct from those afforded by aspirins. Thus, the sulfosuccinimidyl chemistry discussed here provides new opportunity to design additional affinity directed $\beta\beta$ crosslinkers.

Sulfosuccinimidyl chemistry also offers additional advantages over the other acylating bifunctional reagents. The high solubility of the sulfosuccinimidyl esters, particularly as compared with that of the corresponding diaspirins, certainly gives a higher flexibility for the manipulation of the reaction systems. Another advantage in using sulfosuccinimidyl esters is the ease of the synthesis of these reagents, particularly as compared with the synthesis of methyl phosphates. These aspects of sulfosuccinimidyl chemistry certainly provides a higher flexibility for the design of other reagents with increased selectivity.

There is an increased interest in recent years in developing a Hb based oxygen carrier. The transformation of an acellular hemoglobin solution into a blood

substitute, in principle, involves the reduction of the oxygen affinity of cell free Hb to a level comparable to that of HbA in the erythrocytes as well as intramolecular crosslinking of HbA to prevent the dissociation of HbA into $\alpha\beta$ dimers [9, 10]. Many chemical approaches have been developed to achieve these objectives. The recombinant DNA technology that has facilitated the expression of mutant Hb with the desired oxygen affinity in foreign vectors has now changed the emphasis and direction of protein chemists for the preparation of Hb blood substitute. One can now readily envisage a new emphasis for the design of crosslinkers primarily to inhibit the dissociation of the tetramers into $\alpha\beta$ dimers as new mutants of HbA with desired oxygen binding properties and higher stability against oxidative damage are produced by the recombinant DNA technology. An elegant $\alpha\alpha$ crosslink has been indeed designed and introduced into HbA and mutant Hb by genetic engineering. However, the regions accessible for such genetic manipulation appear to be limited and the need for other chemical approaches has been emphasized. Development of new crosslinkers involving new chemistry, the selectivity of which is dictated by the 'affinity' of the reagent to a prechosen domain of Hb could serve as a general inter $\alpha\beta$ dimer crosslinker with a variety of low oxygen affinity Hb variants. The sulfosuccinimidyl chemistry discussed here represents one such general approach.

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A CONVENIENT METHOD FOR THE DETERMINATION OF THE SOLUBILITY OF HEMOGLOBIN AND MODIFIED HEMOGLOBINS.

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ABSTRACT

The removal of a single charged group can drastically alter the solubility of hemoglobin. Alterations in hemoglobin structure to make it of potential use as a blood substitute must produce derivatives which are sufficiently soluble to allow adequate oxygen delivery. We have developed a convenient method for examining modified hemoglobins. How the polymerization of Hemoglobin S is perturbed in the presence of modified hemoglobins is determined. To measure Hemoglobin S polymerization, a rapid temperature jump from $0°$ and a nitrogen atmosphere [1] are not required. In pH 7.4 phosphate buffers at concentrations greater than 2M containing sodium dithionite, dilute solutions (less than 1 mg/ml) of hemoglobin S aggregate at $30°$, or higher, after a delay time (minutes) which depends on the hemoglobin concentration. Light scattering can be used to quantify the extent of polymerization. Chemical modifications of Hemoglobin A can result in altered perturbation of Hemoglobin S polymerization as determined by this method. Modification of hemoglobin to provide suitable oxygen binding characteristics and crosslinking of subunits is a required preliminary to use of hemoglobin as an acellular blood substitute. These modifications often involve the elimination of charged groups from hemoglobin. Information on whether such modifications may have undesirable consequences with respect to solubility properties can be examined using the method described.

INTRODUCTION

The precipitation of Hemoglobin S inside the red blood cell under conditions of low oxygen concentration indicates that very small changes in the functional groups at the surface of the protein can have a large effect on solubility. In the case of Hemoglobin S, the replacement of two residues which are negatively charged in the physiological pH range by two hydrophobic valine residues is sufficient to make the deoxy form of this protein insoluble at the concentrations found inside red blood cells in individuals with sickle cell anemia. Blood substitutes based on Hemoglobin A need to be modified in order to function outside of the red blood cell. Derivatives must be formed to give a suitable oxygen affinity in the absence of the physiological modifier 2,3-diphosphoglycerate (DPG) and to prevent dimer formation and subsequent rapid loss through the kidneys with possible consequent renal damage. Recent efforts to form a suitably modified derivative have used either chemical modification of hemoglobin purified from red blood cells or genetic engineering and production of recombinant hemoglobin. A chemically modified hemoglobin currently being investigated as a potential blood substitute is deoxyhemoglobin crosslinked using bis(3,5-dibromosalicyl) fumarate. A derivative with an appropriate oxygen affinity is formed by the crosslinking of α chains using the lysine 99 residues and resulting in the elimination of two positive charges at physiological pH [2]. A recently produced recombinant hemoglobin contains a glycine residue which crosslinks the C-terminal arginine of one α-chain to the N-terminal valine of a second α-chain resulting in the elimination of a positively charged and a negatively charged group at physiological pH [3]. To be useful as a blood substitute, a hemoglobin derivative has to be sufficiently soluble to allow high enough concentrations of the infused derivative to provide the required oxygen carrying capacity. An indication of solubility may be obtained from the effect of a modified hemoglobin on the polymerization of Hemoglobin S. For example, it has recently been shown that a mutant hemoglobin in which threonine 87 of the

β chain is replaced by an isoleucine residue is much less efficient in inhibiting Hemoglobin S polymerization than is normal Hemoglobin A [4].

The commonly used method of examining the ability of hemoglobins to polymerize measures turpidity of dilute solutions after a rapid temperature jump in high concentration phosphate buffers [1]. Phosphate solutions containing 5 mg/ml sodium dithionite are cooled to 0° in a capped cuvette fitted with a gas inlet and a gas outlet. After flushing the solutions with nitrogen for 15 minutes, an aliquot of cold (0°) hemoglobin is added, mixing is carried out by inversion, the cuvette is warmed in a hot water bath to a predetermined temperature, and then the cuvette is placed in a water jacketed cell holder at the desired final temperature. The optical density at 700 nm is recorded over time. In the present communication, a much simpler method of determining the extent of hemoglobin polymerization as a function of time is described. The method is used to examine the effect of Hemoglobin A and chemically modified derivatives of Hemoglobin A on the polymerization of Hemoglobin S.

METHODS AND MATERIALS

Hemoglobin A and Hemoglobin S were prepared from blood obtained from the Canadian Red Cross. The Millipore Minitan Ultrafiltration System was used to prepare a protein fraction from lyzed red blood cells that passed through a 100,000 nominal molecular weight cutoff filter but was retained by a 30,000 nominal molecular weight cutoff filter [5]. Hemoglobin A was modified with pyridoxal phosphate as previously described [6]. Citrate was covalently bound to the hemoglobin using the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide to activate the carboxyl groups of the citrate.

Aggregation of hemoglobin in phosphate buffers is dependent upon the phosphate concentration and the temperature. At final concentrations of less than 1.0 mg/ml, Hemoglobin S begins to aggregate in minutes at phosphate concentrations greater than 1.0 M using the temperature jump method [1]. Initial experiments indicated that aggregates of hemoglobin S would form at room

temperature when Hemoglobin S was mixed to a final concentration of approximately 1 mg/ml in a 2.42 M potassium phosphate buffer, pH 7.4, containing 10 mg/ml sodium dithionite. To investigate the effects of hemoglobin concentration and temperature on the rate and extent of polymerization, solutions containing potassium phosphate at a concentration of 2.52 M and a pH of 7.4 and sodium dithionite at a concentration of 10 mg/ml were prepared fresh daily. Cuvettes containing 2.4 ml of this solution were allowed to equilibrate for at least 10 minutes in a water jacketed cell holder in a Perkin-Elmer 124 dual beam spectrophotometer. Concentrated hemoglobin solutions in Krebs Ringer bicarbonate to a maximum volume of 0.1 ml were then mixed with the prewarmed phosphate-dithionite solution and the absorbance at 650 nm was recorded. When less than 0.1 ml of hemoglobin was used, the bicarbonate buffer was added to make the total volume added to 0.1 ml.

RESULTS AND DISCUSSION

The increase in light scattering as hemoglobin comes out of solution causes an increase in apparent light absorbance when the absorbance is monitored at 650 nm in a spectrophotometer. Figure 1 shows the result of monitoring absorbance when dilute solutions of Hemoglobin S were converted to the deoxy form in concentrated solutions of potassium phosphate containing dithionite. It can be seen in Figure 1(a) that there is a time delay before polymerization starts which increases as the hemoglobin concentration decreases. Figure 1(b) shows the effect of temperature on polymerization. For a given concentration of Hemoglobin S, the delay time before polymerization starts decreases as temperature is increased. The amount of polymerization as determined from the maximum apparent absorbance increases with increasing temperature. At concentrations greater than 0.15 g/dl polymerization began immediately upon mixing for solutions at 37°. These results appear to be very similar to those reported using the more complicated and cumbersome temperature jump method [1]. Additional evidence that the simplified method produced the same responses was obtained on further analysis
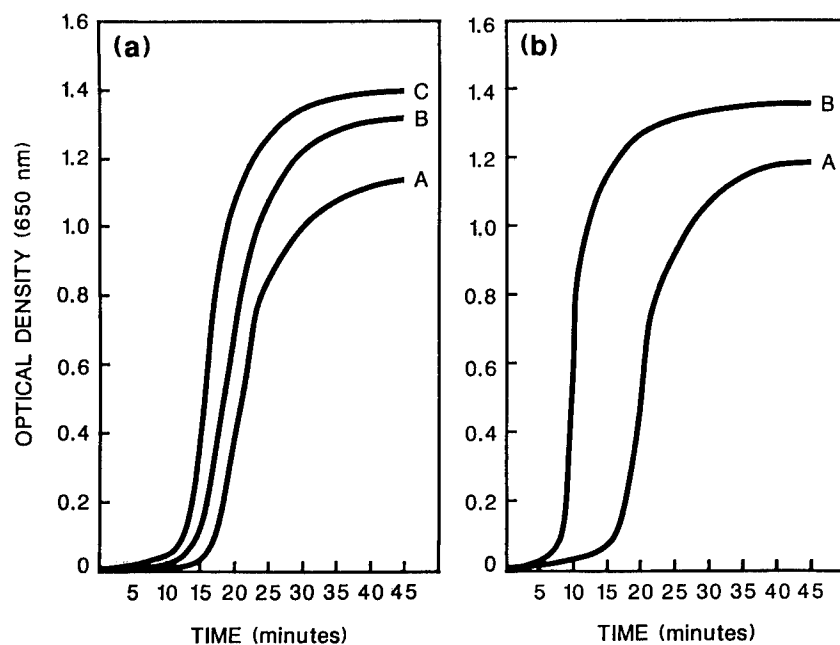
FIGURE 1. The polymerization of Hemoglobin S in potassium phosphate buffer containing dithionite. (a) The effect of increasing concentration on the polymerization at 30°. A - Hemoglobin S concentration 0.12 g/dl, B - Hemoglobin S concentration 0.18 g/dl, C - Hemoglobin S concentration 0.24 g/dl (b) The effect of increasing temperature on the polymerization. A - 0.12 g/dl Hemolgobin S at 30°. B - 0.12 g/dl Hemoglobin S at 37°.

of the data. Figure 2 shows how the delay time for polymerization is determined. Using this definition of delay time, it has been shown that there is a linear relationship between the log of the reciprocal of delay time and the log of concentration when data obtained using the temperature jump method are plotted. Experiments using much higher hemoglobin concentrations in which the onset of gelation as a function of hemoglobin concentration is determined also give a linear relationship when data are analysed in this way [7]. In Figure 3, the delay times for polymerization for the data shown in Fig.1 are plotted. It can be seen a good
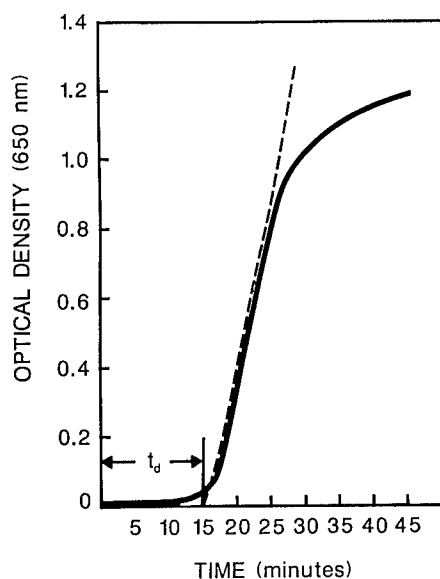
FIGURE 2. Delay time for aggregation. The delay time, t_d, is the time from mixing to the time at which a line representing the maximum slope meets the time axis. Hemoglobin S concentration was 0.12 g/dl and the temperature 30°.

linear relationship is obtained as seen with the other methods. Another indication that the same properties of hemoglobin that were being observed in the temperature jump method were responsible for the development of turpidity in the prewarmed high phosphate solutions was obtained by cooling solutions in which precipitate had been formed in an ice bath. Precipitated Hemoglobin S was found to go back into solution. On warming to 30° the precipitate reformed. Reversible aggregation by changing the temperature is also observed using the temperature jump method [1].

The presence of Hemoglobin A in concentrations of more than 50% prevents the polymerization of Hemoglobin S in the red blood cell under physiological conditions [4]. In the presence of Hemoglobin A, the delay time for the polymerization of hemoglobin S is increased when determined using the
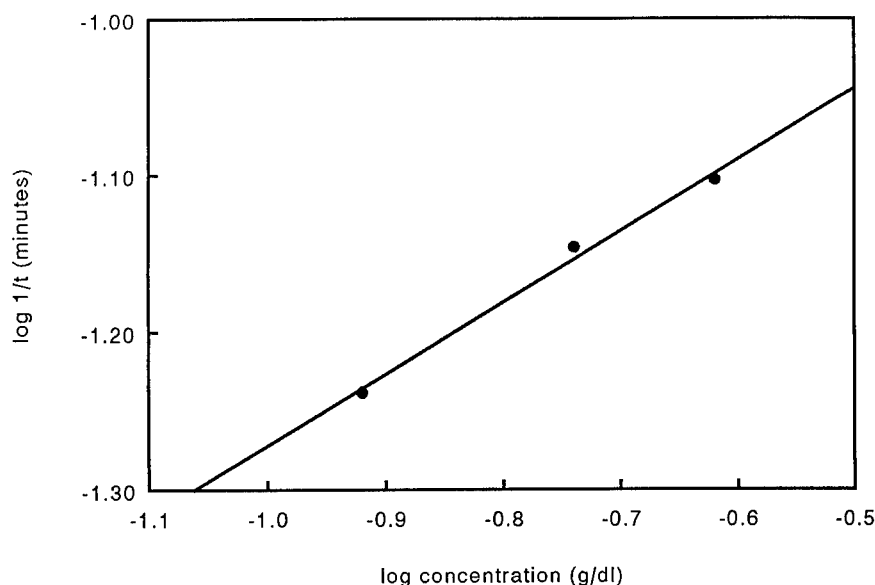
FIGURE 3. Plot of log reciprocal delay time versus log concentration. The data of Fig. 1 is plotted with delay time, t, determined as described in Fig. 2.

temperature jump method. However, the Hemoglobin A participates in the polymerization [8]. Figure 4(a) shows the same effect when polymerization is determined using the simplified method. The delay time for polymerization is increased when Hemoglobin A is mixed with Hemoglobin S, but the increase in optical density indicates that the Hemoglobin A also precipitates. Under identical conditions up to a concentration of 0.4 g/dl there was no detectable precipitation of Hemoglobin A in the absence of Hemoglobin S for at least 1 hour. The effect of a commonly used chemical modification in the preparation of hemoglobin based blood substitutes on the polymerization of Hemoglobin S is also shown in Figure 4(a). Pyridoxalation completely prevents the interaction of Hemoglobin A with Hemoglobin S. The delay time is unchanged and there is no evidence that the pyridoxalated Hemoglobin A participates in the polymerization. It has previously been reported that modification of the DPG binding site of hemoglobin
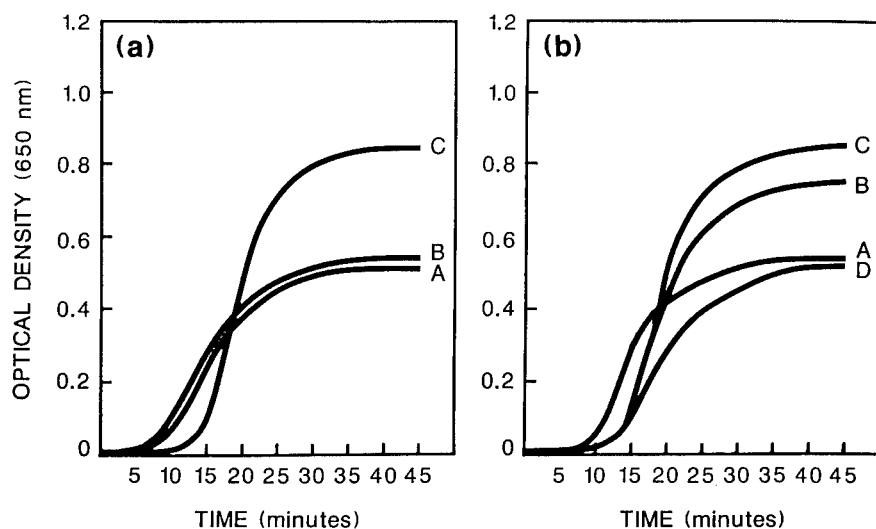
FIGURE 4. Polymerization of Hemoglobin S in the presence of Hemoglobin A and chemically modified Hemoglobin A. All polymerizations were carried out at 37° and the Hemoglobin S concentration was 0.06 g/dl. A) no additions; B) addition of 0.06 g/dl of pyridoxylated Hemoglobin A; C) addition of 0.06 g/dl of Hemoglobin A. b) A) no addition; B) addition of 0.06 g/dl of Hemoglobin A modified for 10 minutes with citrate; C) addition of 0.06 g/dl Hemoglobin A; D) addition of 0.06 g/dl Hemoglobin A modified for 1 hour with citrate.

A with a bifunctional acylating reagent perturbs the interaction of Hemoglobin A with Hemoglobin S and when used to modify Hemoglobin S increases its solubility [9]. The present result suggests that pyridoxylation may have a similar effect.

Introduction of negative charges into a protein by the modification of lysine groups would be expected to increase the electrostatic repulsion and therefore the solubility [10]. Figure 4(b) shows the effect of the introduction of carboxyl groups into Hemoglobin A on the ability of Hemoglobin A to perturb the polymerization of Hemoglobin S. The modification of Hemoglobin A in this way does not decrease the ability to inhibit the onset of polymerization of Hemoglobin S. However, it can be seen that the participation of the modified Hemoglobin A

in the polymerization decreases and that this participation becomes less as the degree of modification increases. This suggests that the Hemoglobin A becomes more soluble as negative charges are incorporated. Modifications of this type may prove to be useful in the preparation of high solubility hemoglobins for use as blood substitutes.

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CONCEPTS OF "TISSUE PO$_2$" IN RELATION TO O$_2$ DELIVERY

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ABSTRACT

Resistance to O$_2$ diffusion is reflected in the difference in pO$_2$ between O$_2$ reservoirs of hemoglobin (Hb) and myoglobin. The very low normal myocyte pO$_2$ (less than one torr but adequate for optimal oxidative ATP synthesis) compared to venous pO$_2$ indicates that blood does not achieve equilibrium with tissue during its passage through capillaries. In the lung, diffusion rate of O$_2$ from alveolus to capillary is normally sufficient to achieve essential equilibrium. However, system-wide capillary pathology and reduced Hb saturation has been observed with distal local ischemia. In peripheral vascular disease (PVD) patients, we found a mean arterial pO$_2$ of 77 torr (normal over 90 torr).

Classical concepts based on "tissue pO$_2$" values derived from venous blood or oxygen electrodes inserted into tissue need re-evaluation. Readings of O$_2$ electrodes moved through tissue range widely from intracapillary levels down toward intracellular levels and do not reflect the pO$_2$ of any particular site. Intravenous pO$_2$ is the result of residual O$_2$ after incomplete diffusion out of capillaries during transit through a tissue, and is not an equilibrium value with some tissue pool.

The effect of HbO$_2$ p50 on oxygen release during the passage of blood through a capillary bed, generally judged on the basis of percentage percent saturation at "tissue pO$_2$", should be judged on the basis of the change in pO$_2$ (the diffusion driving force) associated with a particular degree of HbO$_2$ saturation at a particular p50. The thesis

that O_2 diffusion rate is a major determinant of oxygen delivery is supported by pO_2 responses to treatment of PVD that does not alter blood flow or p50.

INTRODUCTION

Current interest in blood substitutes with oxygen carrying capacity, such as the fluorocarbons and extra-erythrocytic hemoglobin, is aimed primarily at overcoming problems of oxygen delivery from alveoli to tissues. In pursuing this goal, the concept of "tissue oxygen", expressed as mmHg of oxygen tension (Torr) (tpO_2) has proved to be useful, in spite of the fact that tissue consists of capillaries, intracellular, and extracellular components that may differ considerably in pO_2. In the last analysis, the value of greatest interest is the intracellular pO_2 where the oxygen is utilized to generate energy-related molecular species.

This report discusses several concepts that have bearing on the movement of oxygen from within the capillary lumen into the energy utilizing cells.

1. Some structure between the hemoglobin (intraerythrocyte) and myoglobin (intramyocyte) oxygen storage pools offers considerable resistance to oxygen diffusion since there is normally a marked difference in their respective pO_2 [1], [2], [3]. This pO_2 difference indicates that erythrocyte/myocyte pO_2 equilibrium is not reached during the transit of blood through the capillaries. Consequently, oxygen delivery to cells depends not only on perfusion rate, but also residence time of red cells in the tissue [3], which is in turn a function of blood pool size and hematocrit within the tissue capillaries [4].

2. Pathologic changes in tissue capillary structure may influence the passage of oxygen from capillaries into cells. Both local and system-wide disturbances in capillary wall structure are seen in response to local injury, including local ischemia [5].

3. While the rapid establishment of equilibrium between alveolus gases and lung capillary blood in normal subjects is well documented [6], there are data which suggest that capillary pathology may result in failure to reach pO_2 equilibrium [7].

4. In patients whose signs of peripheral vascular disease improve with treatment, but without apparent change in blood flow, a correction of arterial blood oxygen under-saturation also occurs [8].

Methods of estimating tissue pO$_2$

In reviewing the significance of these concepts as they relate to "tissue pO$_2$", the methodology used to estimate tissue pO$_2$ must be considered. Of particular interest is the intracellular pO$_2$ of myocytes, since muscle cells are responsible for over 95% of oxygen consumed. The oxy/deoxymyoglobin ratio is essentially the only available approach to a direct estimation of intracellular pO$_2$. While results by this method vary with conditions, and particularly with pH and temperature, they consistently indicate that intracellular pO$_2$ is normally very low (about 1 torr) [1,2], but still adequate to achieve the optimal rate of mitochondrial ATP synthesis [2].

Other methods of estimating "tissue pO$_2$" do not indicate intracellular levels. On moving a micro oxygen electrode slowly through a muscle, the observed pO$_2$ fluctuates widely from levels under 10 mmHg to typical capillary pO$_2$ values of 30-40 mmHg [9]. While an "average" electrode reading taken to indicate "tissue pO$_2$" can reflect the status of tissue perfusion, it does not give quantitative information about intracellular pO$_2$. The same is true for transcutaneous pO$_2$ (tcpO$_2$) estimates obtained by measuring the pO$_2$ in a chamber above the skin. This pressure is presumed to be in equilibrium with subcutaneous "tissue pO$_2$". Reproducible values, obtained only after local heat induced capillary dilatation, are clinically useful to evaluate perfusion [10], but are not indicative of intracellular pO$_2$.

The pO$_2$ of venous blood draining a particular bed is often presumed to have reached equilibrium with, and to reflect the pO$_2$ of, the tissue drained. While such measurements also have clinical utility, they are not a measure of intracellular pO$_2$.

Difussion from capillary to myocyte

The thin lipid bilayer of capillaries and the high solubility of molecular oxygen in lipids has long been the basis for the assumption that capillary walls offer negligible resistance to oxygen diffusion. This assumption is reinforced by experimental evidence of the speed of equilibration of oxygen tension between alveoli and lung capillaries [6]. Nevertheless, the high pO$_2$ within capillaries compared to the low pO$_2$ in the immediately adjacent myocytes compels the conclusion that intervening structures offer considerable resistance to oxygen diffusion. One commonly neglected component of that intervening structure is the glycocalyx, a polymeric nonlipidic material that coats the capillary endothelial surface and is believed to serve to protect the capillary surface lipids from plasma lipases. The glycocalyx is destroyed by osmic acid fixation (and thus not generally seen in EM studies), but is clearly evident by appropriate

staining [11], and could be a significant component of the barrier to oxygen diffusion from erythrocyte to myocyte. Its experimental destruction in vivo leads to a marked change in capillary hematocrit [4].

A tangential observation relates to the teleologically curious phenomenon that red cells are larger than some of the capillaries through which they can pass only by virtue of their deformability. If the glycocalyx is indeed an oxygen diffusion barrier, its displacement by a tight red cell could permit diffusion directly from red cell to myocyte without disturbing the protective function of the glycocalyx. The occurrence in nature of red cell free oxygen carrying blood under very special conditions (the arctic fish [12]) and the survival of pigs without red cells under high pO_2 conditions [13] confirm direct oxygen diffusion from plasma to cell. It is of interest, however, that such animals survive at plasma pO_2 levels too low to sustain animals with red cells [10]. Is there a change in the glycocalyx barrier?

Effect of Pathology

Under a variety of pathologic conditions, including chronic ischemia, there is capillary wall swelling [14] with an amorphous material of considerable thickness which may further interfere with transcapillary oxygen difussion over and above any effect it may have on capillary blood flow. Peripheral vascular disease patients show a reduced arterial pO_2 compared to normal subjects [7]. This clinical phenomenon is consistent with the observation that rats with one chronically ischemic limb show capillary wall swelling not only in the ischemic limb, but in the other limbs, heart, liver, etc. [14]. Peripheral vascular disease patients treated by an agent described as enhancing oxidative metabolism, with no change in blood flow or hemoglobin p50, showed a parallel improvement in the low ApO_2, elevated blood lactate and signs and symptoms of the affected limb [8]. If capillary wall pathology is a common factor in decreasing alveolus-to-capillary as well as capillary-to-myocyte oxygen diffusion, and the treatment improved reduced trans-capillary oxygen diffusion, one would expect the pattern of responses observed.

CONCLUSION

The above considerations lead to the conclusion that the in vivo evaluation of oxygen carrying blood substitutes should include their influence on hemoglobin-to-myoglobin transport, particularly under conditions of capillary pathology. "Tissue pO_2" considerations are helpful but not sufficient. Tissue pO_2 estimations may go up with improved perfusion, but may go

down with improved capillary-to-myocyte diffusion. Intracellular pO_2 estimations and cell functional capacity are the critical factors for evaluation of potential therapeutic utility.

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HEMOGLOBIN IS A LATE EVENT IN THE DIFFERENTIATION OF FRIEND ERYTHROLEUKEMIC CELLS *IN-VITRO*. I. THE ROLE OF INTERFERON-INDUCED PROTEINS.

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ABSTRACT

Here we report that the interferon (IFN)-induced proteins, 2'-5'-oligoadenylate synthetase (OAS) and IFN-induced protein kinase (PKI), appearance and activity precede that of hemoglobin (Hb) in the differentiation process of Friend erythroleukemic cells (FLC). Since our results are correlative, we assume that OAS and PKI are activated, and act at an early stage in the differentiation process, enabling the late onset of Hb synthesis. It is, thus, suggested that red blood cells harboring specific differentiating genes may be used as more efficient carriers of oxygen-binding molecules.

INTRODUCTION

It is still not clear what is the mechanism responsible for the regulation of gene expression during differentiation. However, growth inhibitory factors may be very helpful in elucidating this mechanism. Interferons (IFNs) are considered to be such factors and therefore represent a possible regulator of cell growth and differentiation. It is now known that part of the IFN-mediated biological effects are due to the action of IFN-induced proteins [1]. One of the best characterized is 2'-5'-oligoadenylate synthetase (OAS), which catalyzes the synthesis of oligomers of adenylic acid in the 2'-5' phosphodiester bond in the presence of double-stranded

769

RNA (dsRNA) and ATP. These oligomers then activate a latent endonuclease (RNAse L) responsible for the degradation of mRNA and rRNA molecules. A second IFN-induced protein is a specific protein kinase (PKI) which depends on dsRNA for its activity. Following autophosphorylation, it is responsible for the phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF-2). Activation of both OAS and PKI regulates protein synthesis, therefore, controlling cell proliferation. Here, we demonstrate that IFN-induced proteins are expressed during erythroid differentiation.

MATERIALS AND METHODS

Cell culture and treatments: FLC were grown at 37°C in DMEM supplemented with 10% fetal calf serum in a humidified incubator enriched with 10% CO_2. To determine *in-vitro* differentiation, we followed the previously described protocols [2].

Preparation of cell extracts and biochemical assays: Cultures were treated as described in the figure legends and immediately thereafter extracts were prepared as previously described [3]. For determination of hemoglobin (Hb) content in cell extracts, the absorption spectrum was recorded either between 340 nm and 700 nm or at 415 nm in a 250 Gilford spectrophotometer. For determination of OAS and PKI activities, cell extracts were assayed as described [3].

Determination of OAS gene expression: Total RNA from cultures was extracted, electrophoresed, blotted, and hybridized with a labeled murine OAS cDNA [3].

Quantitation of bands: Quantitation of labeled bands was performed by scanning autoradiographs in the linear range of exposure with a BioImage System (Millipore).

RESULTS

The appearance of Hb is characteristic of an erythroid differentiated cell. Thus, in this study, after inducing FLC to differentiate, we monitored the appearance of Hb as compared with the expression and activity of IFN-induced proteins. Figure 1 shows the absorption spectrum of a typical cell-induced extract. The net value of the absorbance of extracts prepared from DMSO-treated cells is shown at different wavelengths. Three typical peaks at 415 nm, 540 nm, and 576 nm were clearly seen, increasing with time of induction. The increase in the amount of Hb is a function of time of treatment.
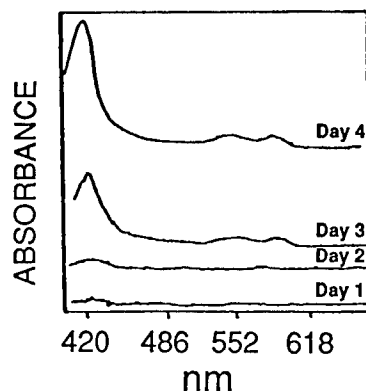
FIGURE 1: **Hemoglobin spectrum**. Cell extracts prepared from untreated and DMSO-treated cells were analyzed in parallel by the Gilford 250 spectrophotometer. The spectrum recorded by untreated samples was automatically subtracted from the value obtained with DMSO-treated cells extracts. The number of days after treatment is indicated on each spectrum.

Based on this result, the appearance of Hb was established after induction with several agents, by recording the absorbance at 415 nm (the major peak) of lysates of cultures as a function of time (Table I). In untreated cells, a small increase in intensity is observed, while after DMSO-induction, a higher increase is observed. Hemin treatment resulted in a moderate increase, but, electrophoretic analysis of the same extracts for the presence of Hb indicated that hemin treatment did not induce the synthesis of a typical Hb molecule (data not shown). We therefore assume that the absorption intensity obtained with hemin treated cells is caused by the hemin content itself, and not by the presence of Hb. IFN-treatment increased only slightly the amount of Hb.

In order to correlate the activation of IFN-induced enzymes during differentiation and appearance of Hb, we first determined the degree of OAS gene expression during the differentiation process, and then, we established in parallel the level of the enzymatic activity under the same experimental conditions. As shown in Table II, the amount of OAS-specific RNA in DMSO-treated cultures reached a peak

LEJBKOWICZ ET AL.

TABLE I: Hemoglobin absorption intensity. Cells were cultured in the absence or presence of either 1.5% DMSO, 40 µg/ml of hemin, or 400 units/ml of β-IFN. Extracts were prepared from treated cells and the absorbance intensity was then recorded at 415 nm.

	ABSORBANCE (415 nm)			
HOURS	24	48	72	96
CONTROL	0.25	0.24	0.39	0.40
DMSO	0.37	0.64	2.24	3.50
HEMIN	0.30	0.55	0.78	0.94
IFN	0.24	0.37	0.56	0.80

TABLE II: OAS gene expression in treated cells. Total RNA was extracted from cells at the indicated times after seeding, and blotted to nitrocellulose filters. Hybridization was performed with ^{32}P-labeled OAS-cDNA probe. The results were quantified by optical densitometry of a 1.7 kb band. ND=not done.

	Relative intensity/40 µg of total RNA						
HOURS	3	6	12	16	24	48	72
CONTROL	0.4	0.8	0.9	0.9	1.1	1.3	2.3
DMSO	1.5	2.4	2.3	2.0	0.6	0.5	0.4
HEMIN	0.08	ND	0.1	ND	0.2	0.2	0.25
IFN	0.6	ND	1.5	ND	2.2	2.4	1.1

level at 6 hours after treatment followed by a gradual decrease up to 16 hours and a sharp decrease thereafter. In contrast, in untreated cells, a gradual increase in the amount of transcripts was observed with time in culture. It should be mentioned that control cells in 96 hours old cultures do not divide under our experimental conditions.

Since hemin failed to induce Hb synthesis in FLC, we studied its effect on the induction of OAS gene expression and activity in order to establish a possible correlation between the IFN-induced proteins and the differentiation process. No difference in the amount of OAS transcripts was seen after hemin treatment, and only trace amounts of transcripts were visible. Furthermore, the results indicate that hemin actually inhibited the increase in OAS gene expression usually observed in crowded untreated-FLC or DMSO-treated cells. In IFN-treated FLC, the largest amount of OAS-specific transcripts appeared at 48 hours after treatment, followed by a reduction thereafter. These kinetics of OAS gene expression are significantly different from that observed with DMSO-treated cells.

Next, we determined the kinetics of OAS enzymatic activity. The 2-5A oligomers synthesized in the biological assay were analyzed by high-voltage electrophoresis on 3MM paper. The radioactive spots representing the different 2-5A oligomers revealed by autoradiographs were cut, and their radioactivity was determined. The results, presented in Table III, demonstrate the level of enzymatic activity. As seen with the OAS RNA transcripts, this level reached a peak activity at 6-12 hours of DMSO-treatment followed by a decrease. On the other hand, in control cells, the enzymatic activity tended to increase with time in culture. In hemin-treated cells the enzymatic activity was completely inhibited in agreement with the results obtained on OAS gene expression. In IFN-treated FLC, the peak activity appeared at 24-48 hours after treatment, followed by a reduction at 72 hours. Still, the relative high level of activity at 72 hours may be due to the elevated level of OAS activity observed in untreated FLC after a few days in culture.

In addition to OAS, another known IFN-induced enzyme is the PKI. As we demonstrated a unique pattern of expression and activity of OAS in our experimental system, it was of interest to study how PKI behaves under the same conditions. The various cell extracts were thus tested for their ability to phosphorylate a 65 kDa protein known to serve as a target for PKI activity. The enzymatic activity was detected by the presence of a labeled band corresponding to 65 kDa. The kinetics of the enzyme appearance (Table IV) is similar to that observed with OAS, namely, in untreated cultures, a gradual increase can be seen with time in culture, whereas a peak at 12 hours and a decrease thereafter is evident in DMSO-treated cells. No induction of PKI activity was evident in hemin-treated cells, in agreement with the lack of response to the induction of OAS gene expression or enzymatic activity in similarly treated cultures. In IFN-treated cells a peak activity was observed at 24-48 hours after treatment, followed by a decrease thereafter.

DISCUSSION

In this study we show that IFN-induced proteins such as OAS or PKI are expressed and activated during the differentiation of FLC in a specific manner. This increased activity appears to occur early after DMSO treatment before the onset of β-globin synthesis which represents the differentiation-specific protein. In fact, when

TABLE III: OAS activity in treated cells. Cells were seeded in the presence of the agents as described in Table I. Cell extracts were prepared, assayed for OAS activity at the indicated times, and the enzymatic products were separated by high-voltage electrophoresis, and radioactivity counted. ND=not done.

	cpmx10^3/20 µg of proteins						
HOURS	3	6	12	16	24	48	72
CONTROL	0.4	0.4	0.5	0.7	0.7	0.9	0.9
DMSO	1.9	2.3	2.2	1.0	0.9	0.3	0.1
HEMIN	0.1	ND	0.1	ND	0.1	0.1	0.1
IFN	0.9	ND	1.6	ND	2.4	3.5	1.1

TABLE IV: PKI activity in treated cells. The extracts prepared from cells at different times after seeding were assayed for the protein kinase activity. The phosphorylated product was analyzed on polyacrylamide SDS gels, autoradiographed and quantified. ND=not done.

	Relative intensity/500 µg of proteins						
HOURS	3	6	12	16	24	48	72
CONTROL	0.2	0.4	0.5	0.6	0.6	0.9	1.6
DMSO	0.6	1.0	2.0	1.1	0.5	0.3	0.2
HEMIN	0.2	ND	0.2	ND	0.2	0.4	0.4
IFN	0.3	ND	0.6	ND	2.4	1.7	0.6

the appearance of β-globin is evident at 48 hours after DMSO-induction, the level of both OAS and PKI is already rather low, indicating that they are no longer needed at this stage of the differentiation process. The increase in the enzymatic activities of OAS and PKI might be due to the release of endogenously-produced IFN as previously shown [4], however, preliminary results with DMSO-treated FLC in the presence of antibodies directed against mouse α/β IFN indicate that OAS activity was expressed and activated to the same level as with cultures in the absence of antibodies (manuscript in preparation). Furthermore, the different kinetics of OAS and PKI activities observed in DMSO and IFN-treated cultures in our study, demonstrate that α/β IFN-release is not involved in the differentiation process. Indeed, an increase in OAS activity in an IFN-independent manner has been demonstrated in other systems [3]. It should be mentioned that hemin failed to induce the synthesis of Hb as well as both OAS and PKI activities, further supporting the notion that the appearance of

these enzymatic activities are correlated to the differentiation process. This seems to indicate that malfunction of IFN-induced proteins may lead to malignancy.

ACKNOWLEDGEMENTS

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REACTION OF HUMAN ENDOTHELIAL CELLS TO BOVINE HEMOGLOBIN SOLUTIONS AND TUMOR NECROSIS FACTOR

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ABSTRACT

Human umbilical vein endothelial cells (HUVEC) were incubated for 24 hours with 0.1mM or 0.3 mM of: [A] unmodified (U) Hb-$Fe^{II}O_2$; [B] UHb-Fe^{III}; [C] UHb-Fe^{IV}-OH; [D] polymerized low molecular weight Hb(<400 kDa); [E] polymerized high molecular weight Hb(<1,020 kDa); [F] polymerized low molecular weight Hb + Endotoxin (2.5 EU/mL); [G] rTNFα 100 pg/mL; [H] rTNFα 400 pg/mL; [I] rTNFα 800 pg/mL. The medium of the incubation was tested for LDH (*index of cell injury*), and for cytokines GM-CSF and IL-1α released by the cells. The data suggests that oxidation status of the iron in the Hb molecule and concentration of Hb play an important role in causing EC injury. The highest toxicity was observed when EC were incubated with 0.1mM of UHb-Fe^{IV}-OH (ferryl-Hb) and no toxicity with 0.3 mM of Hb-Fe^{III} (ferric-Hb). The direct stimulation of EC by Hb for the production of IL-1 was limited, related only to high molecular weight Hb polymers or to Hb+E, however GM-CSF expression was increased by almost all Hb forms. TNF induced dose-related injury (R^2=0.986), and dose-related release of IL-1 (R^2=0.977). A different EC reaction was observed on the release of GM-CSF. Intermediate levels of TNF (400 pg/mL) increased the expression of this cytokine, while high levels (800 pg/mL) blocked its release.

INTRODUCTION

Vascular endothelial cells (EC) and the monocyte/macrophage-leukocyte (M/MØ-L) system are first in line of exposure to injected blood substitutes. Previous work from our laboratory showed that unmodified bovine Hb influences eicosanoid synthesis by the human EC [1]. It was also found that incubation of EC with Hb may increase formation of free oxygen radicals [2]. A more recent study has shown that pure Hb may activate the *in vitro* M/MØ-L system to release tumor necrosis factor (TNF), a cytokine which exerts a variety of effects on EC [3]. The functional properties of EC are especially altered by exposure to TNF and IL-1 [4]. EC are both a target for cytokines and its greatest source. In addition, these polypeptide mediators are involved in initiation of intravascular coagulation, modulation of immune and inflammatory response, regulation of vascular tone, permeability, and hemopoiesis [5-7]. The objective of the present work was to determine whether different Hb oxidative status and Hb biopolymeric forms can mimic TNF-directed cellular injury and stimulate EC to produce other cytokines by the cells.

MATERIALS AND METHODS

Hemoglobin (Hb) preparation and characterization: Bovine unmodified (U) Hb-$Fe^{II}O_2$ and chemically stabilized low molecular weight (M.W.) polymerized Hb (LMWHb) were prepared

according to an earlier developed method [8]. High M.W. Hb (HMWHb) was crosslinked intermolecularly with glutaraldehyde by the procedure of Bonsen et al. [9]. UHb-Fe^{III} and UHb-Fe^{IV}-OH were prepared according to methods published by den Boer et al. [10], and Yamada et al. [11], respectively. LMWHb contaminated with endotoxin (E) was produced by adding 2.5EU/mL of E.Coli 0111:B4 Endotoxin (Whittaker Bioproducts, Inc., Walkersville, MA). Hb solutions dialyzed against Normosol®-R to a final concentration of 1.5 mM were stored at -90°C. Purity of Hb and physical-chemical characteristics were characterized by earlier described quality control methods [12, 13]. The release of iron (Fe) from Hb reacted with H$_2$O$_2$ (molar ratio; 1 : 8) was evaluated by measuring the amount of Fe released following a 15 minutes, 1 and 24 hours incubation period at 37°C. Hb was removed from the reaction by ultrafiltration using a MICROSEP™10K concentrator (Filtron Technology Corp., Northborough, MA). Fe level in TCA-soluble medium was measured by a colorimetric method (Procedure No. 565, Sigma Diagnositcs®, St. Louis, MO).

   Cell culture: Proliferating secondary (2°) cultures of human umbilical vein endothelial cells (HUVEC) were subcultured and grown to confluence in 24-well cell plates (Corning, New York, NY). Experiments were performed on cells which had been passaged by using trypsin-EDTA digestion and treatment with trypsin neutralizing solution. Cells were cultured in sterile, pyrogen free Endothelial Cell Basal Medium supplemented with epidermal growth factor (10 mg/mL), 2% fetal calf serum, gentamicin (0.05 mg/mL) and amphotericin-B (0.05 mg/mL) (Clonetics, San Diego, CA). Cells were characterized by their typical microscopical morphology and human factor VIII secretion.

   Experimental procedure: A monolayer of 140,000 cells/well/0.7ml medium volume was incubated in 5% CO$_2$ at 37°C for 24 hours with 0.1 mM or 0.3 mM of [A] UHb-Fe^{II}O$_2$; [B] UHb-Fe^{III}; [C] UHb-Fe^{IV}-OH; [D] LMWHb (<400 kDa); [E] HMWHb (<1,020 kDa); [F] LMWHb+E (2.5 EU/mL); [G] rTNFα 100 pg/mL (Genzyme Co., Cambridge, MA); [H] rTNFα 400 pg/mL; and [I] rTNFα 800 pg/mL. The medium of incubation was tested for release of lactate dehydrogenase (LDH) using a spectrophotometric method (Procedure No. 228-UV, Sigma Diagnostics®, St. Louis, MO), the level of granulocyte-macrophage colony stimulating factor (GM-CSF) by ELISA technique and IL-1α by RIA method (Genzyme Co., Cambridge, MA). Electron microscopic (E.M.) morphological studies on the cells were also conducted.

   Statistical analysis: The LDH, GM-CSF, IL-1 levels in the medium (n=6) of control, and Hb's or TNF treated cells were compared by a Students t - test. Dose related effects of TNF and Hb's were evaluated by linear regression with simple and polynomial curve fit. The "*goodness of the fit* " of the curves was measured by coefficient of determination (R^2). Analysis was performed by using the StatWorks™ statistical package (Cricket Software, Inc., Philadelphia, PA).

RESULTS

   The unmodified and polymerized Hb solutions used in these experiments are characterized in **TABLE I**. Examination by overloaded isoelectric focusing and thin layer chromatography methods demonstrated the elimination of all non-Hb proteins and phospholipids from the UHb solution. The concentration of endotoxin in final products was 0.08-0.12 EU/mL.

TABLE I. *Characteristics of hemoglobin solutions.*

	$UHb\text{-}Fe^{II}O_2$	$UHbFe^{III}$	$UHb\text{-}Fe^{IV}\text{-}OH$	LMWHb	HMWHb	LMWHb+E
Hemoglobin, mM	1.5	1.5	1.5	1.5	1.5	1.5
Met-Hb (% of Hb)	1.5	89.0	15.0	2.7	7.5	3.0
pH, units	7.38	7.35	7.36	7.40	7.38	7.37
Osmolarity, mOsm/L	295	294	295	298	299	297
Oncotic pressure, mmHg	49.3	49.8	50.1	24.7	21.8	24.6
Viscosity, cP	0.83	0.80	0.81	1.86	2.25	1.87
P_{50}, mmHg	26	-	-	23	20	23
Sodium, mEq/L	139	138	140	140	140	139
Potassium, mEq/L	5.0	4.8	4.9	4.8	4.9	5.0
Chloride, mEq/L	98	99	98	99	98	99
Endotoxin, EU/mL	0.08	0.09	0.09	0.12	0.12	2.5
Phospholipids (TLC)	Absent	Absent	Absent	Absent	Absent	Absent
Non-Hb proteins (IEF)	Absent	Absent	Absent	Absent	Absent	Absent
Mol. Wt., kDa (SEC HPLC)	68	68	68	<400	<1,020	<400

Incubation of Hb with H_2O_2 produced very limited release of Fe. No detectable increase in release of Fe was noted at 15 minutes incubation time. At 1 and 24 hours only 0.01% (0.99 ± 0.09 µg/dL) and 0.02% (1.98 ± 0.08 µg/dL) of Fe was released from Hb, respectively.

Cell injury was indicated by a significant increase in release of LDH to the medium as compared to medium from control EC. Analysis of LDH release revealed large differences in EC injury introduced by various Hb forms (**FIG.1[A]**). The level of LDH was the highest (55.7 ± 5 U/L) following incubation with $UHb\text{-}Fe^{IV}\text{-}OH$, and it decreased in the following order: $UHb\text{-}Fe^{IV}\text{-}OH > UHb\text{-}Fe^{II}O_2 > LMWHb+E > HMWHb > LMWHb$. The Hb solutions in concentration of 0.1 mM introduce less severe injury to EC than in 0.3 mM ($R^2=0.991\pm0.011$). The 0.3 mM of $UHb\text{-}Fe^{III}$ and 0.1 mM of LMWHb did not significantly increase release of LDH as compared to controls. Dose related injury ($R^2=0.986$) was found during incubation of EC with rTNFα (**FIG. 2[A]**). The highest release of LDH was observed following incubation of EC with 400 and 800 pg/mL (30.6 ± 1.8 U/L $p<0.01$ and 40.5 ± 2.7 U/L $p<0.001$, respectively). However, TNF (100 pg/mL) did not introduce any significant changes in LDH level when compared to control (23.4 ± 3.6 U/L vs. 18.0 ± 2.0 U/L).

The LDH release by EC was supported by E.M. findings. Cell damage characterized by the appearance of large cytoplasmic vacuoles, mitochondrial swelling and convoluted nuclei was found when EC were incubated with 0.3 mM of $UHb\text{-}Fe^{IV}\text{-}OH$, $UHb\text{-}Fe^{II}O_2$, LMWHb+E and HMWHb. Normal morphological features of EC were observed after incubation with $UHb\text{-}Fe^{III}$ and LMWHb.

It was found that Hb solutions provide little stimulus for expression of IL-1 (**FIG. 1[B]**). A significant increase in the level of this cytokine was only noted after incubation of EC with 0.3 mM of HMWHb and LMWHb+E ($p<0.01$). The 0.3 mM of $UHb\text{-}Fe^{IV}\text{-}OH$ introduced significant ($p<0.001$) inhibition of IL-1 expression. Non significant changes in IL-1 level were observed after incubation of EC with TNF at a concentration of 100 pg/mL (**FIG. 2[B]**). A dose of 400 pg/mL TNF caused release of 99 ± 16 pg/mL ($p<0.05$), where 800 pg/mL of TNF stimulated production of 170 ± 11 pg/mL IL-1 ($p<0.001$) ($R^2=0.977$).
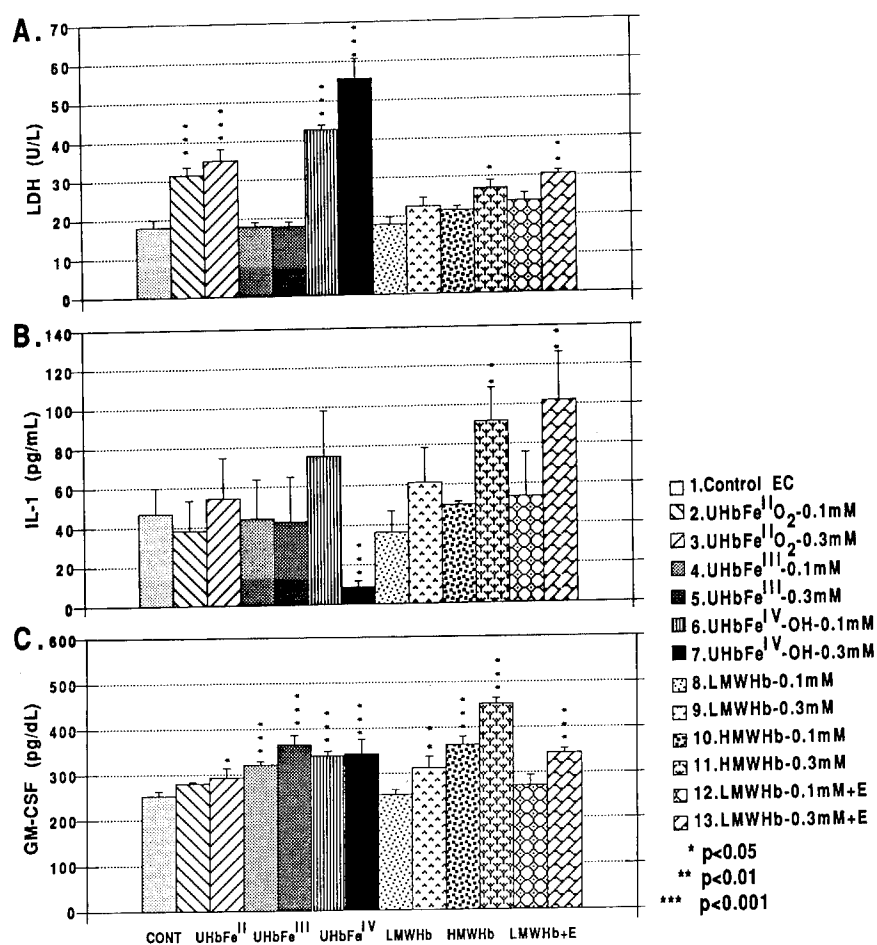
FIGURE 1. Effect of various Hb solutions on EC: **(A)** LDH release, **(B)** IL-1α production, and **(C)** GM-CSF expression. Significance: * p<0.05, ** p<0.01, *** p<0.001.
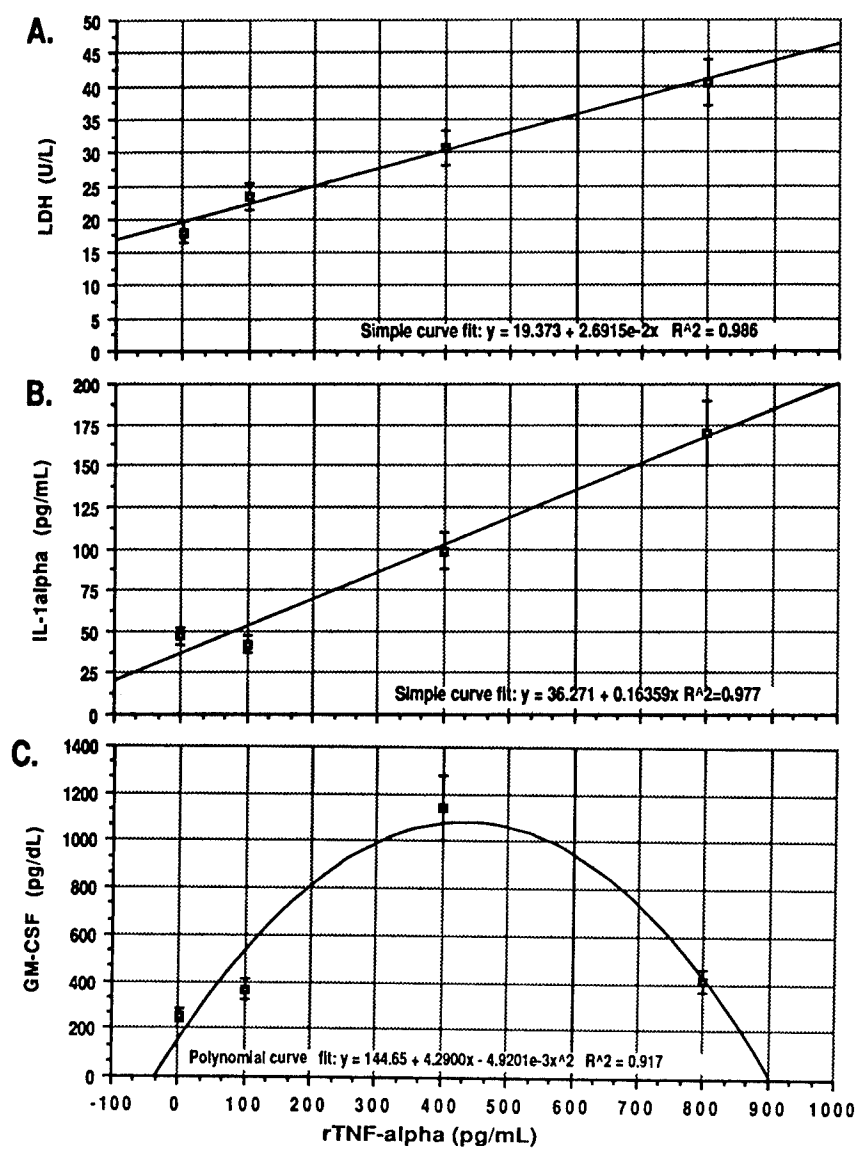
FIGURE 2. Effect of rTNFα (in dose 100, 400, 800 pg/mL) on EC: **(A)** LDH release, **(B)** IL-1α expression, and **(C)** stimulation of GM-CSF production. The *"goodness of the fit"* of the curves was measured by coefficient of determination (R²) (simple or polynomial curve fit).

With regard to the expression of GM-CSF, the level of this cytokine was the highest in response to incubation with 0.3 mM of HMWHb ($p<0.001$) > UHb-Fe^{III} ($p<0.001$) > UHb-Fe^{IV}-OH ($p<0.001$) > LMWHb+E ($p<0.001$) > LMWHb ($p<0.01$) > UHb-Fe$^{II}O_2$ ($p<0.05$) (**FIG. 1[C]**). The Hb in concentration 0.1 mM provided a lesser stimulus. Non significant changes were observed after incubation of EC with 0.1 mM of LMWHb, LMWHb+E and UHb-Fe$^{II}O_2$. A different response of the EC was observed with the release of GM-CSF induced by TNF (**FIG. 2[C]**). The higher TNF level (800 pg/mL) blocked synthesis of GM-CSF. Only the dose 400 pg/mL of TNF significantly increased expression of this cytokine up to $1,142\pm76$ pg/dL ($p<0.001$). This effect of TNF are significant ($R^2=0.917$ by polynomial regression).

DISCUSSION

The data suggests that the oxidation status of iron in the Hb molecule plays an important role in induction of EC injury. To date the most popular explanation of cellular injury introduced by Hb, described in the scientific literature is based on the fact that ferro-Hb (Hb-Fe$^{II}O_2$) may autoxidize to yield ferric-Hb (Hb-Fe^{III}) plus the superoxide anion (O_2^-), which dismutates to hydrogen peroxide (H_2O_2), and in the presence of free iron (Fe^{3+}) may form highly toxic hydroxyl radicals (OH·) [14, 15]. These reactive oxygen species may cause cellular injury; however, it is extremely difficult to explain by these described mechanisms the EC oxidative injury observed in our present work. Besides, production of highly reactive hydroxyl radicals (OH·) from H_2O_2 may occur in the presence of free Fe liberated from heme [16]. According to work from our laboratory, as well as others, release of free iron from Hb molecules following exposure to H_2O_2 is very limited [11]. In fact, in this study no detectable increase in release of Fe was found after 15 minutes, and only 0.02% of the total Fe was released from Hb after 24 hours of incubation.

Due to this fact, it is unlikely that the Fenton or Haber-Weiss reactions are the main mechanisms of endothelial oxidative injury. Our observation that the highest membrane damage was related to the ferryl-Hb (Hb-Fe^{IV}-OH), lower to ferro-Hb and no toxicity following reaction with ferric-Hb, indicates that probably the oxidation of heme iron in Hb-Fe$^{II}O_2$ results in formation of a ferryl-Hb species which directly promotes membrane oxidation, peroxidation, epoxidation [11, 17]. Our previous finding, that the incubation of endothelium with pure ferro-Hb significantly increased the level of H_2O_2, may support the presence of such a mechanism [2]. It was reported that increases in superoxide anion release from EC may occur in response to various stimuli, including cytokines IL-1 and TNF [18]. The dismutation of O_2^- requires enzymatic activity of superoxide dismutase (SOD), which was not present in our experimental model. It was found that certain stimuli, including cytokines may stimulate over-expression of MnSOD by EC, and this enzyme can dismutate O_2^- into H_2O_2 [19], which creates the possibility of the direct reaction of H_2O_2 with extra- or intracellular Hb. Possibly the following reactions which lead to EC damage take place:

[1]　Endothelial Cells + TNF/IL-1/Hb　-------> O_2^-

[2]　Endothelial Cells + TNF/IL-1/(Hb?) -------> MnSOD expression

[3]　O_2^- -------> MnSOD -------> H_2O_2

[4]　H^+ + Hb-Fe$^{II}O_2$ + H_2O_2 -------> Hb-Fe^{IV}-OH + O_2 + H_2O

[5]　Hb-Fe$^{II}O_2$ + H_2O_2 -------> Hb-Fe^{III} + OH· + OH$^-$ + O_2

[6]　Hb-Fe^{IV}-OH + H_2O_2 -------> Hb-Fe^{III} + H_2O + HO$_2^-$

[7]　Hb-Fe^{III} + H_2O_2 + H^+ -------> H_2O + Hb-Fe^{IV}-OH

Because Hb-Fe^{III} in a concentration of 0.3 mM did not show a toxic effect and did not introduce damage to the endothelium, we believe that formation of ferryl-Hb from ferric-Hb in our experimental model has not occurred. Balla et al. [20] reported that prolonged exposure of endothelium to heme or ferric-Hb renders EC remarkably resistant to the oxidant challenge. Those authors concluded that, acutely delivery of free heme to the vasculature is hazardus by sensitizing EC to oxidant damage, while chronic exposure upregulates their defense, by increased production of heme oxygenase and ferritin.

To date, the greates area of investigation has been related to the autoxidation of ferro-Hb and formation of ferric-Hb and intravascular reduction *in vivo* and *in vitro* . However, there is no information about the possibility of the *in vivo* transformation of extracellular ferro-Hb into ferryl-Hb with lipid peroxidative activity [10, 21]. Formation of ferryl-Hb cannot be detected at wavelength 630 ηm [11, 17]. Our unpublished result showed that in some *in vivo* experimental models a moderate increase in ferric-Hb (15-20%) was associated with formation of Hb byproducts with a spectrum similar to that described for ferryl-Hb (λ max = 545 ηm and no absorbance at 630 ηm) and increased level of TBAR-S. This preliminary observation needs to be scientifically evaluated.

An observed lower degree of EC injury by polymerized Hb solutions may be related to a higher oxygen affinity compared to UHb and chemical modification of the Hb surface. It was found that Hb with a higher oxygen affinity (P_{50}: 12 mmHg) produces lower peroxidation of the red blood cell membranes than Hb of low oxygen affinity (P_{50}: 26 mmHg). In addition, COHb did not introduce oxidative damage, similar to that introduced by ferro-Hb [22].

In our experimental model, dose-related increases in LDH release induced by TNF at a concentrations of 400 and 800 pg/ml were probably related to PLA_2 activation, PAF synthesis and changes in membrane permeability by this cytokine [18, 23].

For many years EC were classified as a passive barrier between blood and tissue, non reactive to many blood compounds. Now that basic functions of the EC have been expanded with more knowledge of interactions with cytokines and its expression by the cells, endothelium biological properties and functional status become critical. Reactions mediated by the cytokines are relatively long-lasting (minutes to hours) compared to histamine action (second to minutes). Cytokine production requires stimulation of the proper receptor, changes in gene expression, biochemical synthesis, and its extracellular release [4, 5]. It is well documented in the scientific literature that EC are both a source and a target for cytokines. The TNF and IL-1 molecules are considered the most potent cytokines in modulation of EC function. These cytokines mediate PAF synthesis, activate PLA_2, stimulate prostaglandin and leukotriene formation, induce EDRF/Nitric Oxide formation, stimulate synthesis of endothelin, facilitate thrombus formation, induce von Willebrand factor,increase permeability of EC to macromolecules, stimulate production of more IL-1, IL-6, IL-8, G-SCF, M-CSF, GM-CSF, express the adhesion molecules ELAM, VCAM and ICAM, and facilitate together with IL-4, production of the monocyte chemotactic proteins (MCP) [4-6, 23-25]. By its production, activated EC participate in inflammatory and thrombotic reactions, immunity and hemopoiesis. It was also found that EC are a great source of growth factors such as G-CSF and GM-CSF, which participate in the regulation of erythropoiesis [7, 26].

To date there is no information relating Hb to activation and production of biologically active cytokines by EC. However, it was reported that purified HbAo could stimulate the EC

ACTIVATED MONONUCLEAR PHAGOCYTES AND LYMPHOID CELLS

DIRECT STIMULUS:
- ENDOTOXIN (LPS)
- Hb+LPS
- Hb + STROMAL LIPIDS
- HIGH M.W Hb POLYMERS
- UNMODIFIED PURE HbFe-II
- FERRYL HbFe-IV

TNF and/or IL-1

- PAF SYNTHESIS
- ACTIVATION OF PLA2
- STIMULATION OF PROSTAGLANDIN AND LEUKOTRIENE FORMATION
- SUPEROXIDE ANION AND MnSOD RELEASE
- INDUCTION OF EDRF/NO PRODUCTION
- STIMULATION OF ENDOTHELIN SYNTHESIS
- FACILITATION OF THROMBUS FORMATION
- INDUCTION OF WILLEBRAND (vW) FACTOR
- INCREASE OF PERMEABILITY TO MACROMOLECULES
- STIMULATION OF PRODUCTION: IL-1, IL-6, IL-5 and HEMATOPOIETIC GROWTH FACTORS: G-CSF, M-CSF, GM-CSF
- EXPRESSION OF ADHESION MOLECULES: ELAM, VCAM, ICAM
- PRODUCTION OF MONOCYTE CHEMOTACTIC PROTEINS (MCP)

-EDRF/NO INHIBITION
-FREE OXYGEN RADICALS GENERATION
-LIPID PEROXIDATION
-OXIDATIVE CELL INJURY
-CHANGES IN CYCLOOXYGENASE PATHWAY OF ARACHIDONIC A.
-INCREASE OF PROCOAGULANT ACTIVITIES OF EC
-EXPRESSION OF ADHESION PROTEINS
-GM-CSF & ERYTHROPOIESIS
-GM-CSF OVER PRODUCTION MAY RELEASE HISTAMINE FROM BASOPHILS
-IL-1 SYNTHESIS

ENDOTHELIAL CELLS
SMOOTH MUSCLE

FIGURE 3. Theoretical model of indirect and direct activation of endothelium by Hb solutions. According to Mantovani et al., Pober et al., Broudy et al., Bussolino et al., Goldblum et al., Rossi et al., Visner et al., Matsubara et al., McFaul et al., Lane et al., Feola et al., and Simoni et al.

to bind mononuclear leukocytes and caused significantly greater neutrophil polarization and chemotaxis [27]. On the other hand, it was found that exposure of EC to perfluorocarbon emulsion did not alter the up-regulation of ICAM or ELAM in response to IL-1 and TNF, while ICAM up-regulation in response to LPS was inhibited [28].

Our experiments have shown that cellular injury introduced by Hb is not correlated with IL-1 and GM-CSF expression, which suggests that the mechanisms of oxidative injury and activation of EC to release cytokines are different. McFaul et al. found that catalase, indomethacin, pentoxifylline or desferrioxamine did not prevent EC stimulation by HbAo, or release by human mononuclear leukocytes of polarizing and chemotactic substances, suggesting that the Hb stimulatory activity probably is not based on the oxidative stress theory [27].

Production of IL-1 by EC was found to be more pronounced when Hb was in a concentration of 0.3 mM. The increase in IL-1 expression by EC was related only to HMWHb and LMWHb+E. Published inducers of IL-1 by vascular cells include LPS, TNF and IL-1 itself [5]. Studies by Waage et al. suggested a positive correlation between high serum levels of IL-1 and death [29]. Elevated levels of IL-1 or TNF were found in ECMO, burns and septic shock

patients [29-31]. Biological and metabolic effects of IL-1 are correlated with cardiovascular collapse during septic shock [32]. Nevertheless, the EC source of IL-1 may be secondary; elevated circulating levels of this cytokine from vascular cells may introduce systemic and morphological changes. High M.W. Hb polymers have been shown to be a stimulus for IL-1 expression. In our present experimental model, only a direct effect of the large Hb polymers on IL-1 receptors may be considered as explanation of this synthesis, because low polymeric forms and the native Hb tetramer did not introduce such activation. Due to the fact that the lipid-A portion of endotoxin stimulates the production of IL-1, observed changes in EC activity during incubation with contaminated Hb by LPS are normal [33].

The secretion of GM-CSF by EC was found to be affected by all forms of Hb. Hb in concentration 0.3 mM activated endothelium to produce more GM-CSF. The highest expression of this cytokine was observed after incubation with high M.W. Hb, ferric-Hb, and Hb+E. GM-CSF is essential for the proliferation and differentiation of erythroid precursor cells [5-7, 26]. In higher concentrations, it may release histamine from the basophils, increase neutrophil-endothelial cell adhesion by activating the adhesion receptor (LAM-1), and promote leukocyte migration into inflammatory areas [34].

The vascular endothelial cells produce colony stimulating factors (CSF), as well as IL-1 and IL-6, which have CSF activity, all of which are involved in the regulation of hematopoiesis [5-7, 35]. It was reported that overproduction of IL-1 and TNF inhibits hematopoietic precursor cell proliferation and directly inhibits the expression of erythroid burst-forming units [36,37]. The hematological abnormalities include peripheral leukopenia and hypoproductive anemia [38]. Previously reported stimulatory effects of Hb on hemopoiesis has not been substantiated [39]. Preliminary work from our laboratory suggests that purity, polymeric status and Hb concentration may affect erythropoietic capability. We believe that studies related to hematopietic activity of Hb must include more investigations of the cytokines role, because their expression by various Hb's is clearly documented [3].

In our experimental model TNF was shown to be an effective stimulator of IL-1 expression by the EC in a dose-related manner. The effect of TNF on colony stimulating factor synthesis indicates that a moderate dose may activate GM-CSF production, but high TNF (800 pg/mL) levels introduced inhibition of its expression. This *in vitro* observation may help in understanding the mechanisms related to an inhibitory effect by elevated TNF levels on hematopoietic growth factor activities and suppression of the granulocyte-macrophage progenitor cells [36-37]. However, it was reported that IL-1 and TNF in low concentrations may support directly or indirectly the erythropoietic effect [7, 26, 36]. Furthermore, the balance between the levels of hemopoietic stimulators seems to be of critical importance in the regulation of hemopoietic progenitor cell proliferation, as well as other reactions at the cell and organ level.

Nevertheless, Hb was found to activate expression of the cytokines (especially GM-CSF) from the endothelial vascular cells. We believe that this direct stimulation is no greater than the indirect one, mediated by TNF, which can be released by pure Hb from the activated M/MØ-L system (FIG.3).

CONCLUSIONS

1. The oxidation status of the iron in the Hb molecule, plus the concentration of Hb plays an important role in EC oxidative injury. In this study, ferryl-Hb produced the most toxic effect, while ferric-Hb showed no toxicity. Mechanisms of oxidative stress introduced by Hb can no longer be explained solely by the Fenton and Haber-Weiss reactions. More scientific attention must focus on a role of ferryl-Hb.

2. Hb was found to influence cytokine synthesis by EC. The greatest changes were observed in the expression of GM-CSF. Almost all Hb forms influenced synthesis of this cytokine. Increase in IL-1 expression by Hb was limited, and related only to large Hb polymers and Hb contaminated with endotoxin. Hb at a concentration of 0.3 mM produced more significant changes than 0.1 mM.

3. A Hb-induced interference with GM-CSF expression by EC may help explain the erythropoietic effect produced by Hb. However, future studies are needed.

4. TNF was shown to be a potent activator of EC function. It was found that a high dose of this cytokine may inhibit expression of GM-CSF by EC, while small and moderate doses possess a stimulatory influence.

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SIGNIFICANT PROLONGATION OF GUINEA PIG HEART CONTRACTION TRANSPLANTED IN RAT AFTER REMOVAL OF ANTI-XENO-ANTIBODIES BY WHOLE BODY RINSE-OUT(WBRO) WITH HEMOGLOBIN SOLUTION.

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ABSTRACT

Purpose : In order to investigate effectiveness of removal of the anti-xeno- antibodies in xenotransplantation(xeno Tx), WBRO using pyridoxalated-human hemoglobin-polyethyleneglycol conjugate (PHP solution) was performed prior to transplantation(Tx) of a guinea pig heart in a rat.

Materials & Method : Experiment I. Removal of the immunoglobulins and the anti-guinea pig lymphocytotoxic antibody (ALA) by WBRO. Exchange transfusion with the PHP solution was done in the Tx-expected rats until a hematocrit lowered below 5% (n=11). Experiment II. Xeno heart Txs. Guinea pig hearts were transplanted into rats without immunosuppressants 1) without (n=8) or 2) with the WBRO (n=8).

Results : Experiment I. Levels lowered to 14 % in IgG, 17 % in IgA and 6% in IgM, respectively, to initial values after the WBRO. An ALA titer lowered from 4 X (+) to 1 X (-) after the WBRO. Experiment II. An average heart contraction period was 10.4±1.8 minutes 1) without the WBRO in contrast to 472.5±4.8 minutes with the WBRO (p<0.01).

AGISHI ET AL.

Conclusion : WBRO using PHP solution is effective in removal of the anti-xeno-antibodies and consequent prolongation of survival of the xenograft.

INTRODUCTION

Transplantations have been postulated as an ideal modality of treatment for the functionally-failed organs. However, the most serious and difficult problem is a shortage of the donor grafts. Therefore, xenotransplantations (xeno-Txs) have been collecting an interest as a possible solution to solve the problem. Severe hyperacute rejection which inevitablly occurs in discordant combinations must be overcome for the successful xeno-Txs.

It is a generally accepted recognition that the naturally acquired xenoantibodies (xeno-Abs) existing in the recipients plays an important role in triggering the hyperacute rejection. This investigation aims to evaluate usefulness of removal of the xeno-Abs by the WBRO with PHP solution and its effects on prolongation of a graft survival period.

MATERIALS AND METHODS

1.Experimental Animals:

Male Lewis-strain rats weighing 200 - 300 gms were used as the recipients while Hartlay-strain guinea pigs weighing 200 - 250 gms were as the donors.

2.Removal of the Xeno-Abs (Experiment I) :

The xeno-Abs were removed by the WBRO which is a modified exchange transfusion using PHP solution [1)2)3)]. For this purpose, the rats anesthetized with ether were exanguinated via the femoral vein and infused with PHP solution via the jugular vein at a rate of 15 - 20 ml/hr until a hematocrit became lower than 5%. It took approximately 3 hrs (Figure 1).

3.Transplantations (Experiment II) :

According to the Ono-Lindsey's technique, the heart transplantation was done [4)]. The hearts extirpated from the guinea pigs were flushed with and

Lewis rat 250-300g
$\begin{pmatrix}\text{blood volume} & \text{12-18ml} \\ \text{Ht} & \text{43-46\%}\end{pmatrix}$

infusion of PHP solution
15-20ml/h (total 30-60ml)

miniroller pump

exanguination

PHP:pyridoxylated Hb polyoxyethylene conjugate
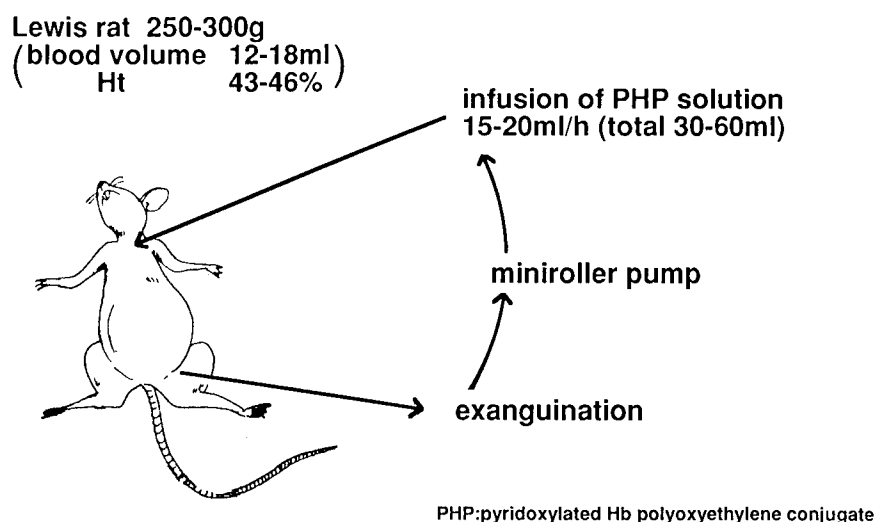
Figure 1.

Rat Whole Body Rinse-out with PHP Solution

stored in 4 degree centigrade heparin added physiological saline until
transplantations. The graft aorta was anastomosed to the recipient
abdominal aorta and the graft pulmonary artery was anastomosed to the
recipient inferior caval vein both in an end-to-side fashion. An ischemic
time was between 25 and 30 minutes. A total operation time was between
40 and 50 minutes.

Experiment II-1) ; Xeno-Tx was done without the WBRO (n=8).

Experiment II-2) ; Xeno-Tx was done after the WBRO (n=8).

No immunosupressants were administered in both xeno-Tx experiments.

RESULTS

Experiment I :

Immunoglobulins Levels ; Levels lowered to 14% in IgG, 17% in IgA and
6% in IgM in an average to an initial value, respectively, immediately

after the WBRO. These levels tended to recover as to be 25% in IgG, 60% in IgA, and 70% in IgM 7 days, and approximately 100% in all immunoglobulins 14 days after the WBRO(Figure 2).

Anti-Lymphocytotoxic Antibody Level ; An average level of the anti-guinea pig lymphocytotoxic antibody lowered from 4 × (+) to 1 ×(-) after the WBRO. However, the anti-guinea pig hemagglutinin antibody was not detected in the rat blood even before the WBRO.

Experiment II :

Experiment II-1) ; All transplanted hearts started contracting immediately after releasing the vascular clamps, and gradually became cyanotic and dark. The hearts became standing-still in 10.4 ±1.8 minutes in an average after the clamp release. Experiment II-2) ; All transplanted hearts maintained normal appearance longer compared to Experiment II-1) and kept contracting as long as 472.5±74.8 minutes in an average after the clamp release(Table I).

DISCUSSION

Xeno-Tx is seemingly a promising approach in the current situation in which difficulty in obtaining the donor grafts will never be easily solved. It is a generally accepted concept that the hyperacute rejection, which is irreversibly severe, and frequently initiates immediately after xeno-Tx, is triggered by an interaction between the xeno-Abs and the substances existing in endothelial cells of the transplanted graft. However, clinical experiences in ABO-blood-type-incompatible kidney transplantations which are assumed to be a model of concordant xeno-Tx, indicate that preceding removal of the anti-A and/or B antibodies leads such transplantations successful[5) 6)].

According to our previous observation that over 90 % of protein-bound molecules could be effectively removed, the WBRO using PHP solution as applied to remove the xeno-Abs in a discordant heart Tx model between guinea pig and rat. The experimetal results evidenced that levels in the immunoglobulins and the anti-guinea pig lymphocytotoxic antibody were remarkably reduced by this procedure, and consequently,
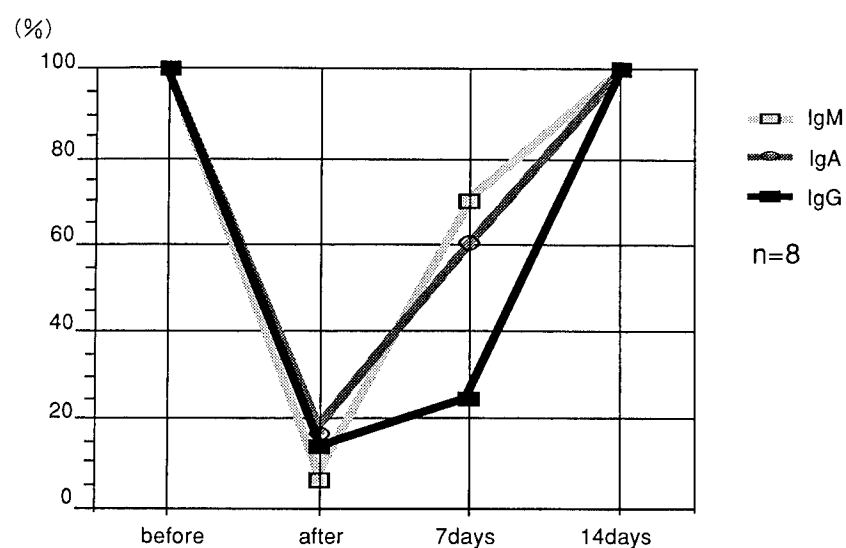
Figure 2.

**Change in Immunoglobulin Levels
by Whole Body Rinse-out with PHP Solution**

Table 1.

Contraction Period of Transplanted Hearts

Experimental group	Graft survival time (in minutes)	Mean graft survival time (Mean±SD)
Xeno Tx Without WBRO n=8	12,10,8,10 10,8,13,12	10.4±1.8 ※
Xeno Tx with WBRO n=8	480,360,420,480 480,600,420,540	472.5±74.8 ※

※ P<0.01   WBRO : whole body rinse-out

an average survival period of the transplanted hearts prolonged from 10 minutes to 472 minutes without any other immunosuppressive measures. In conclusion, the WBRO using PHP solution is effective on an initial suppression of the hyperacute rejection in the xeno-Tx by removing the xeno-Abs.

ACKNOWLEDGEMENT

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PEG-HEMOGLOBIN:AN EFFICIENT OXYGEN-DELIVERY SYSTEM IN THE RAT EXCHANGE TRANSFUSION AND HYPOVOLEMIC SHOCK MODELS.

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ABSTRACT

Polyethylene glycol-hemoglobin (PEG-Hb is a purified bovine hemoglobin molecule modified by polyethylene glycol. Oxygen delivery to the tissues, in rat exchange transfusion and hypovolemic shock models, was studied to determine whether the oxygen-carrying capacity of PEG-Hb is as efficient as red blood cells.

OBJECTIVES

This study was undertaken to investigate the oxygen-carrying capacity of polyethylene glycol-modified hemoglobin (PEG-Hb) in vivo by direct measurement of oxygen tension in tissues. The animal models used were designed as possible predictors of performance in human clinical applications.

A phosphorescence imaging technique, in which a measured phosphorescence lifetime is converted into absolute oxygen tension [1,2,3], was used. Palladium-porphyrin, injected into the circulation, is quenched by molecular oxygen unloaded by hemoglobin, resulting in phosphorescence decay. This method allows continuous measurement of tissue oxygen tension over a relatively wide area (1cm x 1cm) for the lifetime of the probe.

PROTOCOLS

Sprague-Dawley rats weighing 250 - 350g were anesthetized with sodium pentobarbital. Pd-porphyrin was injected into the tail vein at 20 mg/kg (1ml/kg).

Bolus Injection

In the bolus injection model, 6% PEG-Hb was infused via tail vein at 0.2ml/min, to an overload of 5ml/kg. Tissue oxygen tension was monitored for 5 minutes and an additional 10ml/kg of PEG-Hb was then infused. Oxygen tension was measured in abdominal muscle, liver, and kidney.

Hypovolemic Shock

Hypovolemic shock was induced by stepwise bleeding from the femoral vein. Ten ml/kg, 7ml/kg, 5ml/kg, and 3ml/kg were drawn at 15 minute intervals, to a total of 25ml/kg over one hour. Fifteen minutes after final bleeding, resuscitation was begun with the infusion of 25ml/kg of one of the three replacement fluids; 1) Ringer's lactate, 2) autologous blood, or 3) 6% PEG-Hb. Tissue oxygen tension was monitored throughout the study and for 10 minutes after resuscitation.

Exchange Transfusion

Rats were bled from the femoral vein and immediately infused with equivalent volumes of Ringer's lactate or PEG-Hb, until 30% blood volume replacement was achieved. Oxygen levels were measured in abdominal muscle tissue.

RESULTS

In the bolus infusion model the oxygen tension in rat abdominal muscle increased by about 10% after an injection of 5ml/kg of PEG-Hb, and by 35% after injection of an additional 10ml/kg (Figure 1). Kidney tissue showed an increase in oxygen tension of 33% after the 5ml/kg injection and 61% after the additional 10ml/kg was injected (Figure 2). Liver showed the greatest increase in oxygen levels with a 93% increase after 5ml/kg of PEG-Hb and 142% after a total of 15ml/kg (Figure 3). Since liver and kidney are more highly vascularized than muscle tissue, oxygen delivery is greater to these organs.

In the hypovolemic shock model, tissue oxygen tension was measured in abdominal muscle. Animals resuscitated with Ringer's lactate recovered only 11 - 25% of pre-shock tissue oxygen levels (Figure 4). When autologous blood was the resuscitation fluid, oxygen tension recovery was 70 -125% of pre-shock levels
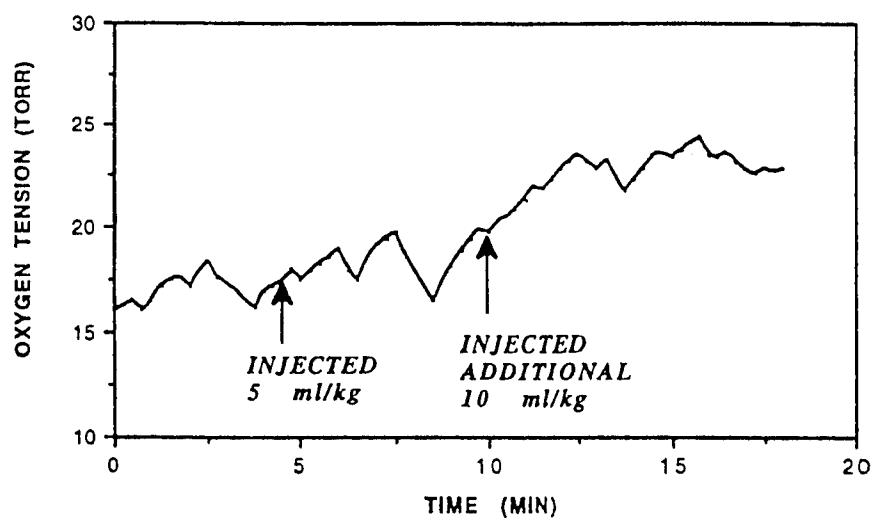
FIGURE 1.   OXYGEN TENSION IN RAT ABDOMINAL MUSCLE AFTER BOLUS INJECTION OF PEG-HB.
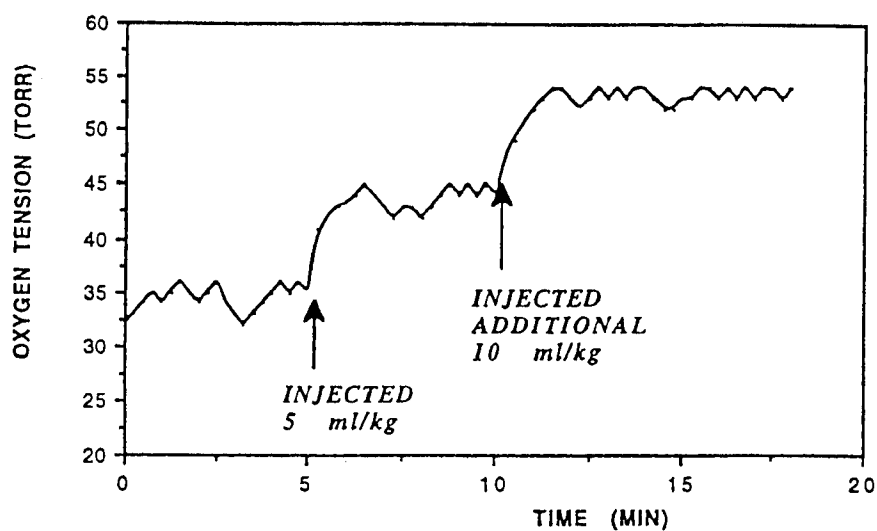
FIGURE 2.   OXYGEN TENSION IN RAT KIDNEY AFTER BOLUS INJECTION OF PEG-HB.
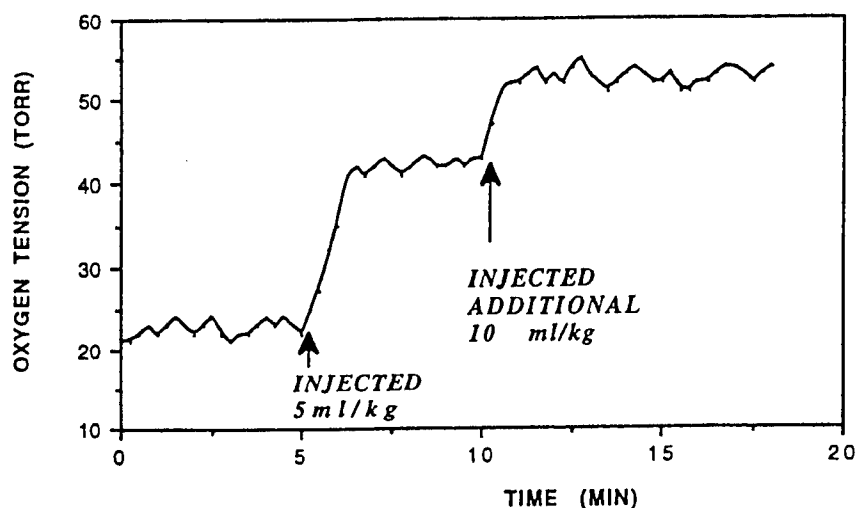
FIGURE 3.   TISSUE OXYGEN TENSION OF LIVER AFTER BOLUS
            INJECTION OF PEG-HB.

(Figure 5). PEG-Hb resuscitation resulted in 118 -150% tissue oxygen recovery
(Figure 6).

In the 30% exchange transfusion model, tissue oxygen tension was measured in
abdominal muscle. Exchange transfusion with Ringer's lactate evidenced a 90%
decrease in tissue oxygen in the rat (Figure 7), while exchange transfusion with
6% PEG-Hb as the replacement fluid showed a return to normal tissue oxygen
levels (Figure 8).

CONCLUSION

PEG-Hb has shown excellent oxygen-delivering capability in all models in this
study. Bolus infusion of PEG-Hb, even at the 5ml/kg level, produced
hyperoxygenation of the liver and kidney, with tissue oxygen tension increases
of 93% and 33% respectively. PEG-Hb, as the replacement fluid in both the
hypovolemic shock and exchange transfusion rat models, delivered oxygen to the
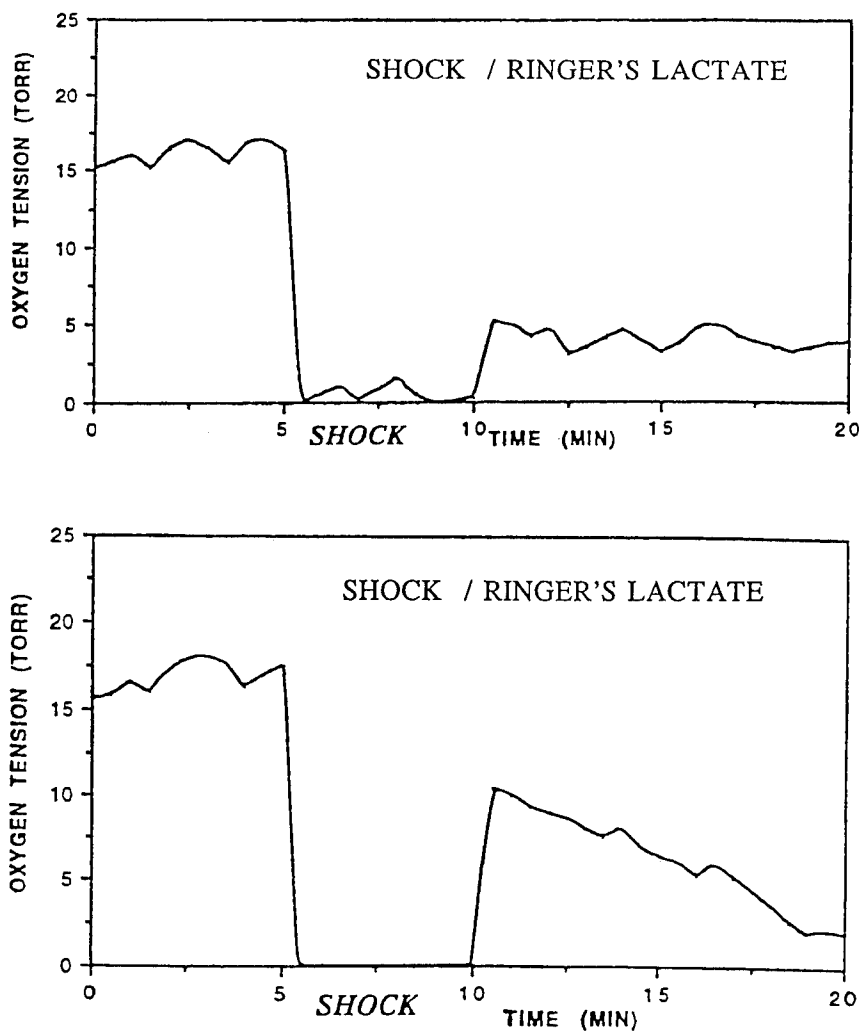
FIGURE 4.   OXYGEN   TENSION   IN   ABDOMINAL   MUSCLE   AFTER
HYPOVOLEMIC SHOCK RESUSCITATION WITH RINGER'S
LACTATE.
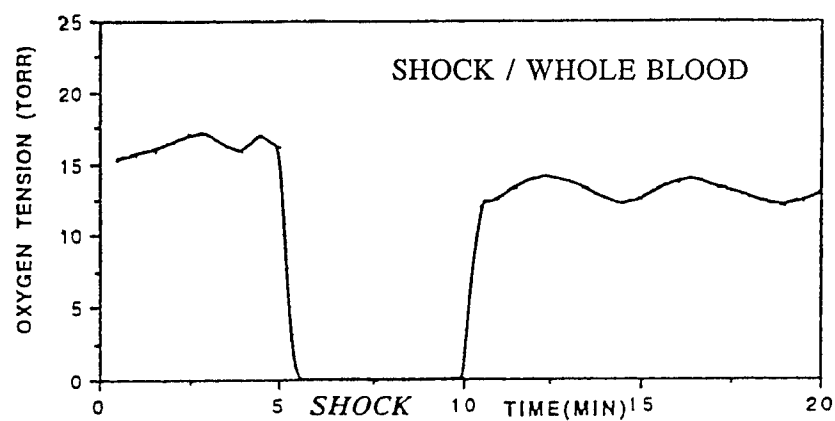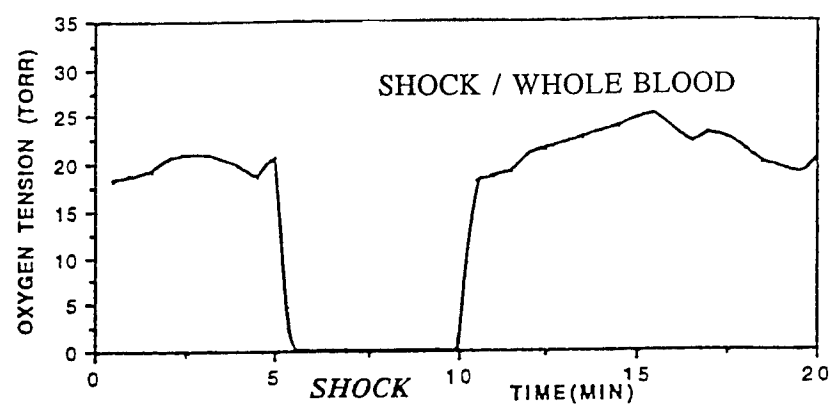
FIGURE 5. OXYGEN TENSION IN ABDOMINAL MUSCLE AFTER HYPOVOLEMIC SHOCK RESUSCITATION WITH AUTOLOGOUS BLOOD.
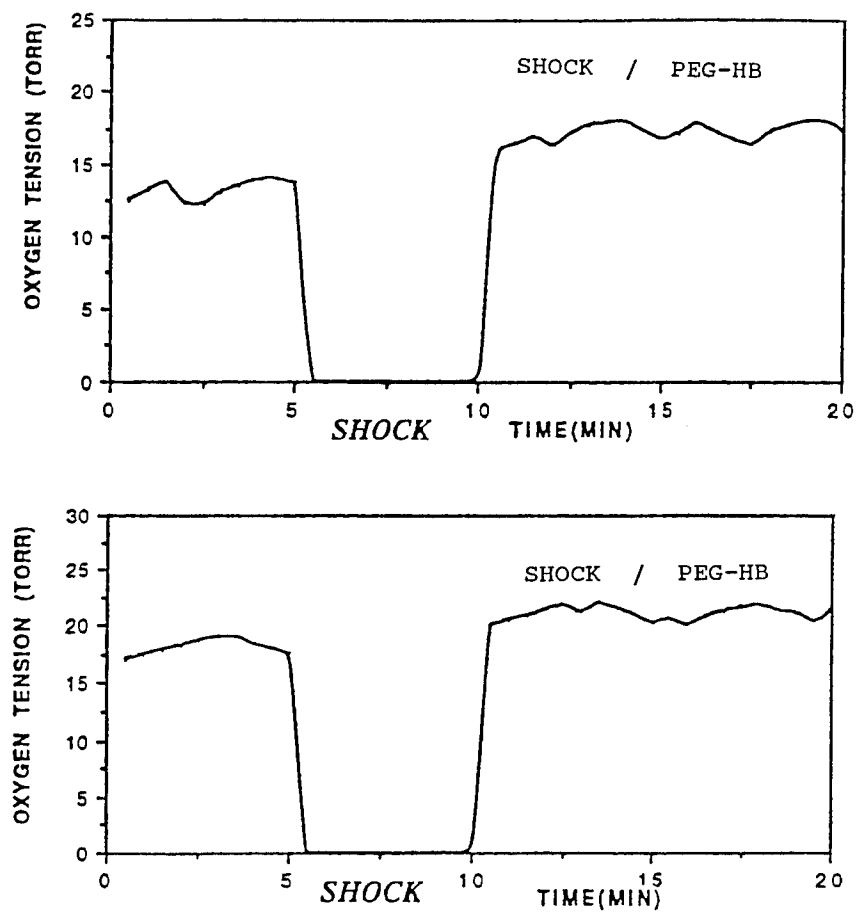
FIGURE 6. OXYGEN TENSION IN ABDOMINAL MUSCLE AFTER HYPOVOLEMIC SHOCK RESUSCITATION WITH PEG-HEMOGLOBIN.
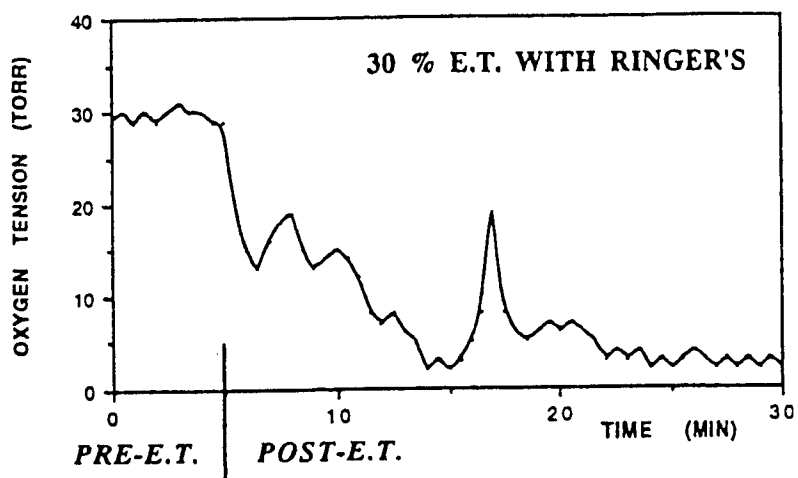
FIGURE 7.   OXYGEN TENSION IN ABDOMINAL MUSCLE AFTER 30%
            EXCHANGE TRANSFUSION WITH RINGER'S LACTATE.
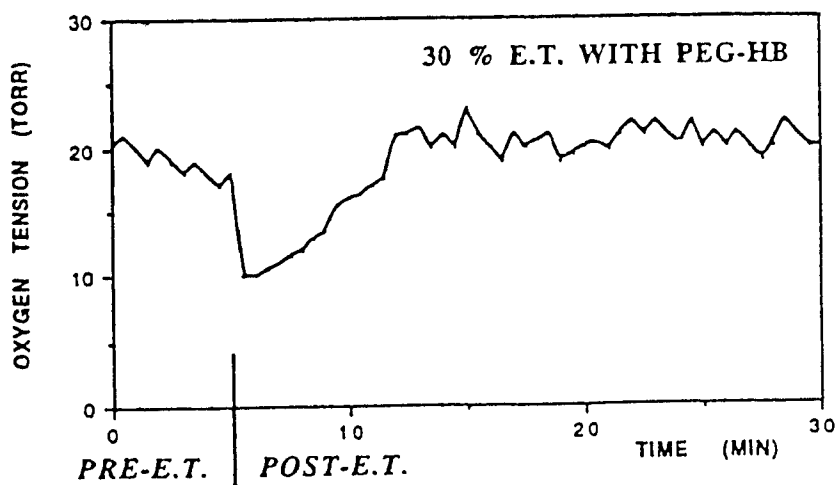
FIGURE 8.   OXYGEN TENSION IN ABDOMINAL MUSCLE AFTER 30%
            EXCHANGE TRANSFUSION WITH PEG-HB.

monitored tissues up to, or exceeding normal levels. This suggests that 6% PEG-Hb solution has oxygen-delivering properties equivalent to, or better than whole blood as a replacement fluid.

The oxygen-carrying capability of PEG-Hb and its ability to unload the oxygen to the tissues, combined with its long half-life (19 hours in rats, 42 hours in dogs), may offer therapeutic potential in a clinical setting [4].

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NORMAL OXYGEN TENSION RESTORED IN THE ISCHEMIC RAT LIVER MODEL BY PEG-HEMOGLOBIN.

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ABSTRACT

Cell damage initiated during ischemia, as a result of oxygen depletion, continues during reperfusion, and recovery is dependent on the length of the ischemic period. This study investigates the effect of polyethylene glycol-modified hemoglobin (PEG-Hb) on recovery of tissue oxygen tension after induced ischemia.

INTRODUCTION

We have examined the performance of polyethylene glycol-modified hemoglobin (PEG-Hb) as an oxygen carrying fluid during ischemia of parenchymal organs. Native bovine hemoglobin was obtained from red blood cells, purified to homogeneity, and conjugated to succinimidyl PEG. The degree of conjugation was controlled to maintain the P_{50}, viscosity, and colloid osmotic pressure at levels similar to that of human blood. Protein purity of greater than 99% was determined by HPLC and isoelectric focusing [1].

Oxygen tension in the blood vessels of the liver of rats was measured noninvasively, using a phosphorescence imaging technique (2). A phosphorescent oxygen-dependent probe, injected into the bloodstream of the test animals, is photoactivated, the phosphorescence lifetime of the probe is

measured and using the Stern-Vollmer equation, converted to oxygen tension. Oxygen tension was measured by Oxyspot (Medical Systems Corp., Greenvale, NY).

PROCEDURE

Sprague-Dawley rats (260-325g) were anesthetized with sodium pentobarbital. Approximately 4mg of palladium meso-tetra-(4-carboxyphenyl) porphyrin (Pd-Tetra) (Porphyrin Products, Logan, UT), as a solution of 8.5mg/ml in 0.9% NaCl with 60 mg/ml bovine serum albumin, fraction V (ICN Immuno-Biologicals, Costa Mesa, CA), at pH 7.4, was injected into the tail vein of both test and control animals. Exchange transfusions were performed on the test animals via catheterization of the femoral vein with 30% of blood volume replaced with 6% (g/dl) PEG-Hb. Controls were not transfused. Those animals that were exchange transfused received an additional 2.0 mg of Pd-Tetra after the transfusion to replace any of the phosphorescent probe lost in the transfusion.

Ischemia was induced by clamping of the hepatic artery, the hepatic vein, and the portal vein, for 30 minutes. Oxygen concentration in the liver was measured before and after ischemia.

Blood samples were taken immediately before, and up to 4 hours after ischemia was induced, to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels .

RESULTS

Tissue Oxygen Levels

As shown in Figure 1A, the first control rat showed almost no recovery of normal tissue oxygen within the 30 minute observation period following

A

ISCHEMIC PERIOD

B
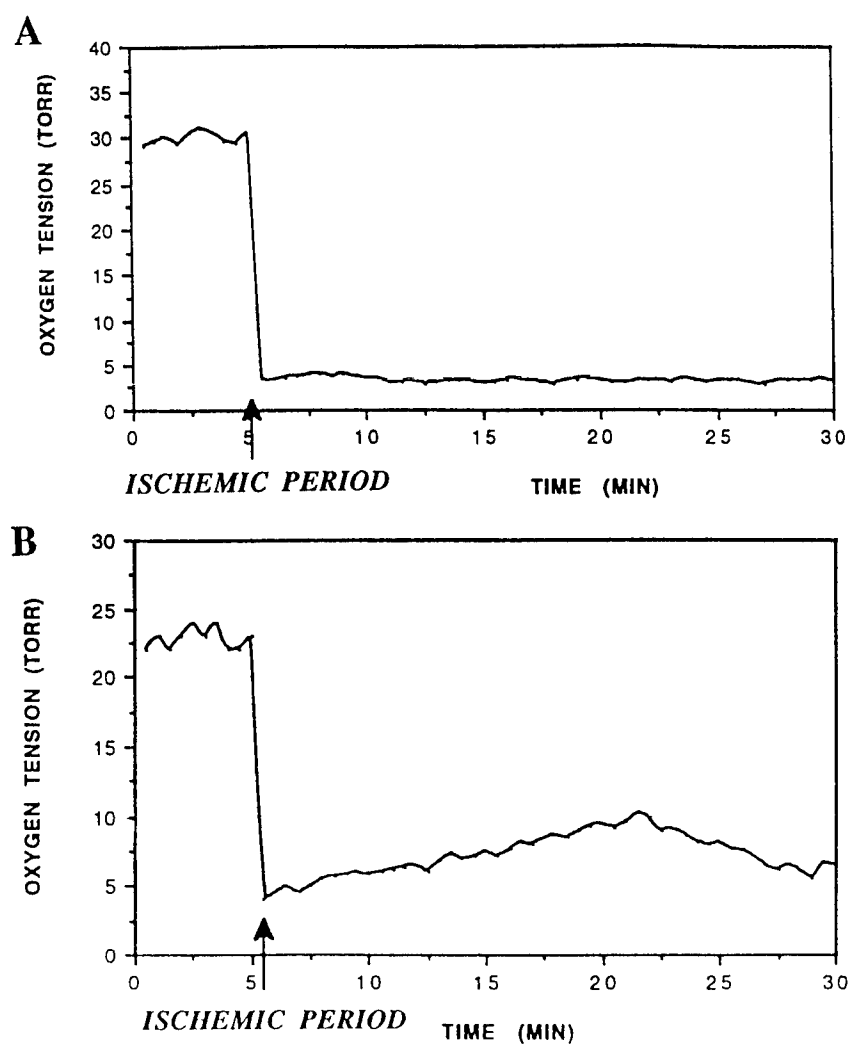
ISCHEMIC PERIOD

FIGURE 1. OXYGEN TENSION IN LIVER OF CONTROL RATS AFTER 30 MINUTE ISCHEMIA.

ischemia. Oxygen tension in the liver was 29-31 torr before ischemia, dropped
to 3-4 torr during the ischemic period, and remained at 3-3.5 torr during the
post-ischemic period.

The second control animal (Figure 1B), showed a small recovery in tissue
oxygen, to about 10 torr, after 21-22 minutes, however, the oxygen tension
dropped back to 6-8 torr by 28-30 minutes.

Oxygen tension in the liver parenchyma of the first 6% PEG-Hb pre-exchange
transfused rat (Figure 2A) was 23-28 torr before induction of ischemia,
dropped to 5 torr during the ischemic period, and slowly increased to 16-17
torr by 21-23 minutes, where it plateaued and held through the 30 minute
observation period. Recovery of tissue oxygen was 65%.

Figure 2B shows the data for the second pre-exchange transfused rat. Oxygen
concentration in liver tissue dropped from 22-27 torr, to 2 torr during
ischemia, recovering back to 15-16 torr. The recovery in tissue oxygen for this
animal was 63%.

Liver Enzyme Levels
Figure 4 represents the plasma levels of liver enzymes in control and PEG-Hb
transfused animals pre-, and up to 4 hours post-ischemia, at 1 hour intervals.
The plasma levels of the enzymes increased in both groups.

DISCUSSION
The 30 minute ischemic period was adopted for this study since it seemed a
reasonable estimate of duration of clinical ischemia before treatment.
Ischemias of 3, 5, 8, and 15 minute duration were also tested, and found to
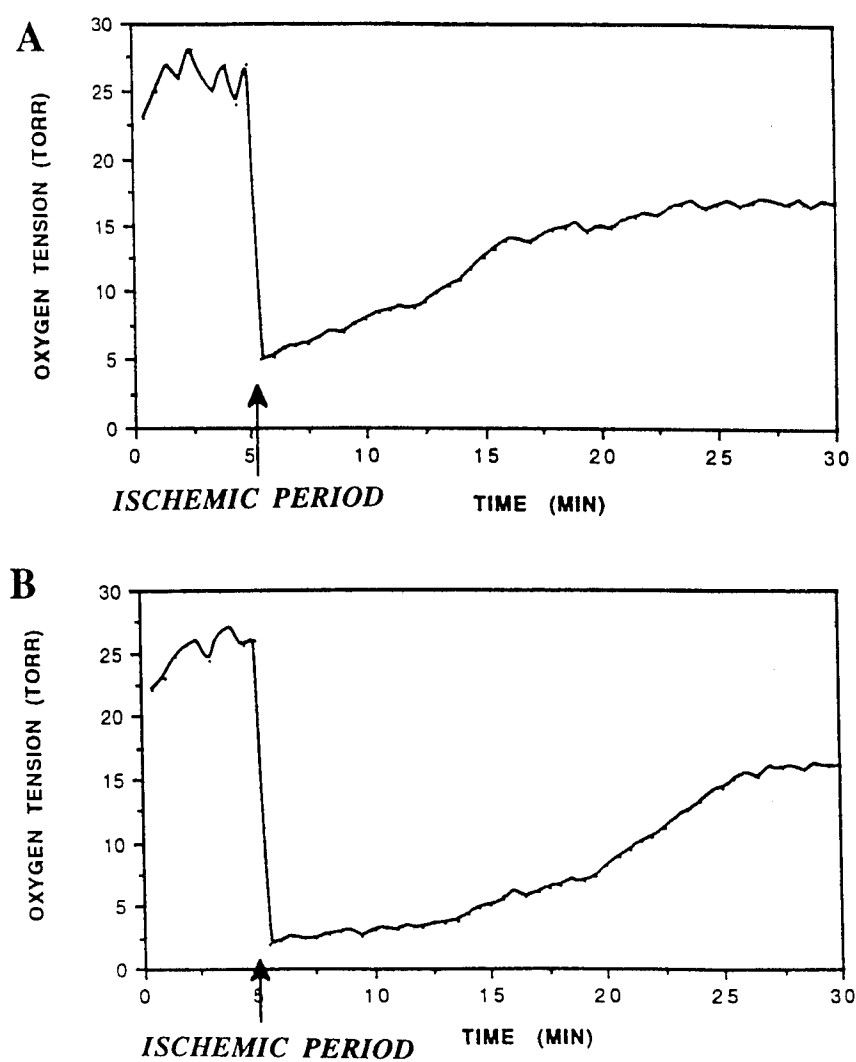
FIGURE 2. OXYGEN TENSION IN LIVER OF PEG-HB RATS AFTER 30 MINUTE ISCHEMIA.

A

B
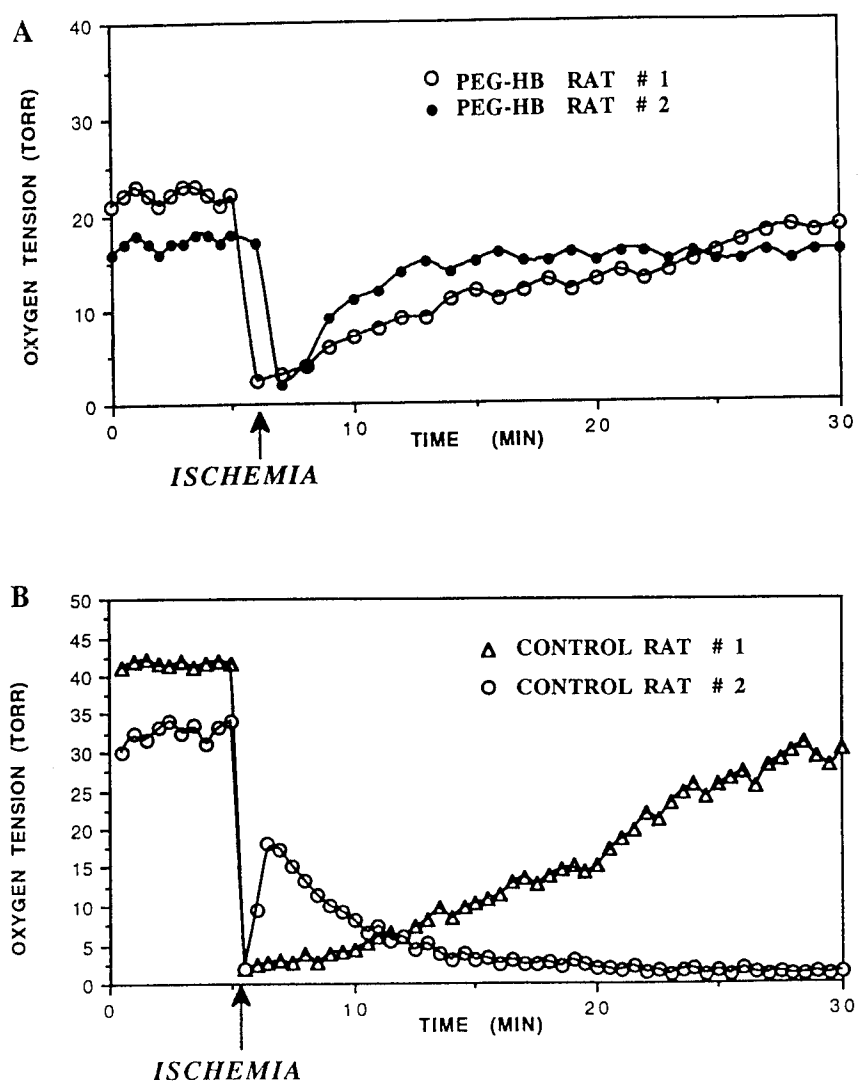
FIGURE 3. OXYGEN TENSION IN RAT LIVER AFTER 3 MINUTE
ISCHEMIA.
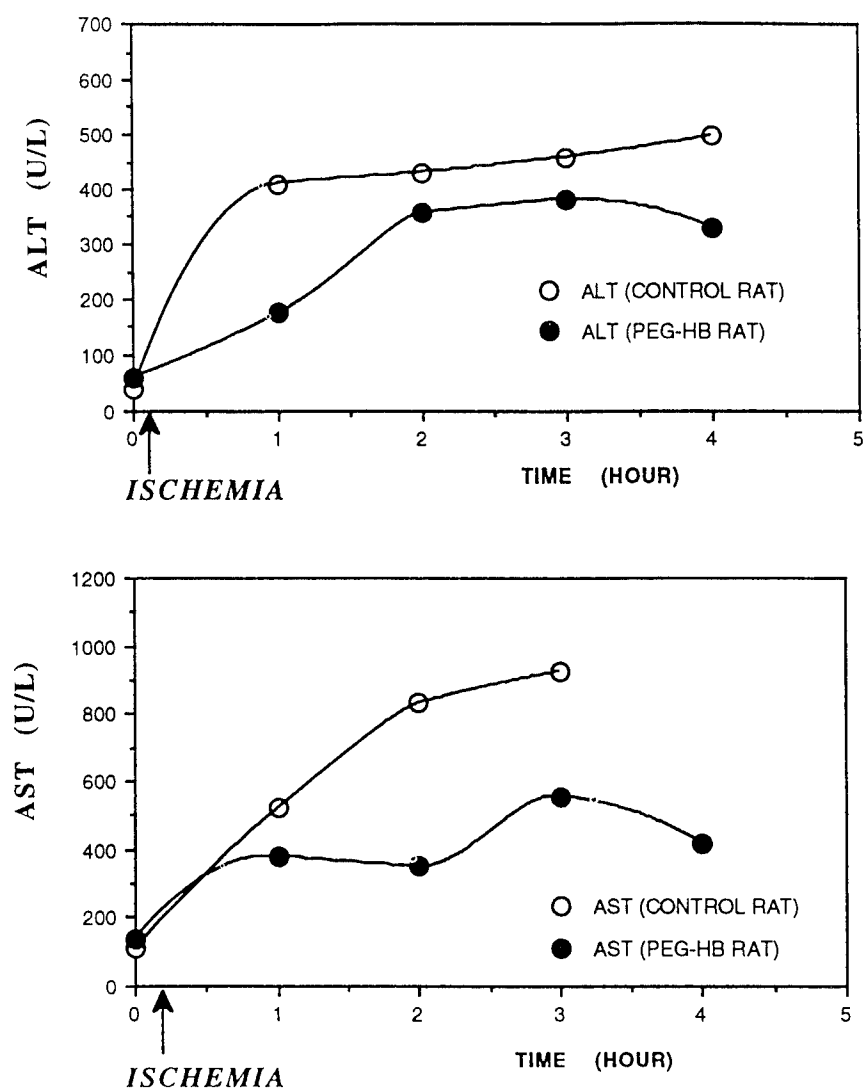
FIGURE 4. ALT AND AST IN RAT LIVER ISCHEMIA.

produce confusing and unreliable results. Figure 3B illustrates the variable

response of control animals to 3 minute ischemia. Figure 3A demonstrates a

return to normal oxygen tension in PEG-Hb pre-transfused rats.

Post-ischemic tissue oxygen recovery has been shown to be considerably better

in 6% (g/dl) PEG-Hb pre-exchange transfused (30% blood volume) rats than

in the controls. More studies need to be done to ascertain that this protection

against ischemic damage is due solely to the presence of PEG-Hb in the

vasculature.

The appearance of AST and ALT in the serum is a standard clinical marker of

liver damage (3). Our data show an increase in enzyme levels for both the

controls and the PEG-Hb transfused groups.

CONCLUSIONS

In the ischemia models presented in this study, PEG-Hb appears to help the

hepatic cells recover more quickly from hypoxia and delay the onset of

ischemic cell damage. Further studies are planned to clarify the potential for

PEG-Hb in the treatment of ischemia.

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DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb): EFFECT OF HEMODILUTION DURING FOCAL CEREBRAL ISCHEMIA IN RATS

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ABSTRACT

The efficacy of hemodilution therapy, to ameliorate cerebral ischemia, is limited by an accompanying decrease in oxygen content. We assessed the effect of hemodilution, with diaspirin cross-linked hemoglobin (DCLHb), on cerebral blood flow (CBF) and infarct after middle cerebral artery occlusion (MCAo).

Rats (n = 36) were alloted to a control group in which hematocrit (Hct) was not manipulated, or reduced with DCLHb to 30% (30/DCLHb), 16% (16/DCLHb), or 9% (9/DCLHb). After MCAo, the brain area with a CBF of 0-10 ml· 100g^{-1}· min^{-1} was determined. This area was decreased in the 30/DCLHb and 16/DCLHb groups vs the Control group; and was less in the 9/DCLHb group vs the other groups.

Different rats (n = 49) were hemodiluted with DCLHb or Albumin (Alb): Control, 30/Alb, 30/DCLHb, 16/DCLHb, or 9/DCLHb. After 3-hr of MCAo and 2-hr of reperfusion, infarct area was determined. Brain infarct was less in the 30/DCLHb and 16/DCLHb groups vs the Control and 30/Alb groups; and was less in the 9/DCLHb group vs the other groups.

The results of this study support the hypothesis that hemodilution with DCLHb decreases cerebral ischemia in a dose-dependent manner, and in terms of brain ischemia is a more proficient hemodiluting fluid than albumin.

INTRODUCTION

As hematocrit decreases, there is a logarithmic decrease in viscosity and increase in CBF (1). Accordingly, hemodilution has been advocated as a treatment of focal cerebral ischemia (2). Although laboratory evaluations of hemodilution in animals models of cerebral ischemia have generated positive results (3-6), the results of human trials have been inconsistent (7-13). Although many reasons may account for this diverse response (14,15), one explanation with merit is a decrease in oxygen content and inherent limitation in oxygen transport when non-oxygen binding fluids are employed. Thus, an increase in oxygen transport induced by augmenting CBF may be negated by a concurrent decrease in oxygen carrying capacity. As the steepest portion of the viscosity-hematocrit curve is at hematocrits >30% (16), hemodilution therapy has been restricted to modest reductions in hematocrit. It is proposed that if hematocrit reductions are limited to this range the benefit from an increase in CBF will exceed the disadvantage from reduced oxygen content, and oxygen delivery will increase. Accordingly, the magnitude of therapy in previous trials has been limited.

α-α diaspirin cross-linked hemoglobin (DCLHb) is a molecular hemoglobin solution with a viscosity similar to albumin (1.3 centistokes [17]) but with oxygen carrying capacity and without hypotensive properties that are present with most hemodilution fluids. Accordingly, we evaluated the dose-related effect of hemodilution with DCLHb on CBF, after 10-min of MCAo in rats. In addition, we compared the effect of hemodilution with DCLHb versus albumin on brain injury and edema during temporary MCAo in rats.

MATERIALS AND METHODS

Part A: Prior to MCAo Spontaneously Hypertensive Rats (n=54) were hemodiluted to one of the following hematocrits (Hct):

44/Hct. Blood volume increased by giving 8.0 ml of donor blood (Hct not manipulated).

37/Hct. Blood volume and Hct (37%) manipulated by 8.0 ml of DCLHb (7g·dl⁻¹, Baxter Healthcare Corporation; Deerfield, IL).

30/Hct. Blood volume and Hct (30%) manipulated by a 5.0 ml exchange transfusion with DCLHb, and an 8.0 ml topload of DCLHb.

23/Hct. Blood volume and Hct (23%) manipulated by a 10.0 ml exchange transfusion with DCLHb, and an 8.0 ml topload of DCLHb.

16/Hct. Blood volume and Hct (16%) manipulated by a 15.0 ml exchange transfusion with DCLHb, and an 8.0 ml topload of DCLHb.

9/Hct. Blood volume and Hct (9%) manipulated by a 20.0 ml exchange transfusion with DCLHb, and an 8.0 ml topload of DCLHb.

Via a subtemporal craniectomy, MCAo was achieved with 10-0 monofilament nylon suture, in two locations, to achieve consistent ischemia to both cortical and sub-cortical tissue. CBF was determined with ^{14}C-iodoantipyrine (18). Coronal brain sections were evaluated for the area with a CBF of 0-10 ml·100g⁻¹·min⁻¹.

Part B: Spontaneously Hypertensive Rats (n=49) were given DCLHb (10 g·dl⁻¹) or albumin (10 g·dl⁻¹) in an identical manner as Part A. However, in this part MCAo was achieved for 180-min followed by 120-min of reperfusion: 1) **Control,** 2) **30/Alb,** 3) **30/DCLHb,** 4) **16/DCLHb,** or 5) **9/DCLHb.** After MCAo and reperfusion, infarct size was analyzed with 2,3,5-triphenyltetrazolium chloride, and edema by microgravimetry (19,20).

The data was assessed by ANOVA.

RESULTS

Part A: There was no difference between the 44/Hct and 37/Hct groups. However, for the other four groups, this CBF area was less as hematocrit decreased (see Table I).

Part B: Infarct size was less in the 30/DCLHb and 16/DCLHb groups versus the Control and 30/Alb groups; and was less in the 9/DCLHb group versus the other four groups, see Table II. Specific gravity was less (increased brain

Table I-area (mean±SD) of 0-10 ml· 100g⁻¹· min⁻¹ CBF. Reported as a percent of the hemisphere ipsilateral to MCAo. *p<0.05 versus the 44/Hct and 37/Hct groups. †p<0.05 versus the other five groups.

	44/Hct	37/Hct	30/Hct	23/Hct	16/Hct	9/Hct
CBF	21±6	19±6	12±3*	13±6*	13±5*	7±3†

Table II-area (% of the cross-sectional area for the hemisphere ipsilateral to MCAo, mean±SD) of infarction. *p<0.05 versus the Control and 30/Alb groups. †p<0.05 versus the other four groups.

	Control	30/Alb	30/DCLHb	16/DCLHb	9/DCLHb
Infarct	42±4	38±3	27±4*	22±5*	18±3†

water) in the Control group (1.035±0.03) versus the 30/Alb (1.041±0.003), 30/DCLHb (1.042±0.001), 16/DCLHb (1.043±0.002), and 9/DCLHb (1.042±0.02) groups (p<0.05).

DISCUSSION

The results of hemodilution studies in humans have been inconsistent (7-13). This inconsistency may be related to at least four issues. The first concerns the possibility of a transient interval following the onset of ischemia in which maneuvers that augment CBF are effective in limiting injury (3-5,8-13). If therapy is instituted following this "window of opportunity", ischemic injury may have matured to a point at which therapeutic efficacy is absent and only detrimental side-effects are manifest (14,15).

The second issue concerns potential hypotensive effects of hemodilution therapy (4,8,9). If decreases in perfusion pressure occur during

critical phases of ischemia, when minor reductions in perfusion pressure produce meaningful decreases in CBF, the beneficial rheologic effects of hemodilution may be concealed. Hemoglobin substitutes are known to increase blood pressure, possibly by binding nitric oxide (21), thereby maximizing the efficacy of hemodilution therapy.

The third issue concerns the optimal hematocrit reduction. In human isovolemic hemodilution studies with a negative result, hematocrit reductions were on the order of 5-10% (8,9). The available evidence suggests that, when using non-oxygen binding fluids, a reduction of the hematocrit to ≈30% provides optimal oxygen delivery to ischemic areas of the brain (16). The final, and related, issue concerns inherent limitations in oxygen delivery when using non-oxygen binding fluids for hemodilution. In such a scenario, any increase in oxygen delivery effected by hemodilution-induced increases in CBF would be limited or nullified by an associated decrease in oxygen carrying capacity. In addition, the magnitude of hematocrit reductions are limited in that when hematocrit is <30%, although CBF continues to increase, absolute oxygen delivery is reduced secondary to decreased oxygen carrying capacity.

In summary, these results support a hypothesis that hemodilution decreases focal cerebral ischemic injury, and when an oxygen binding fluid is used there is a dose-dependent effect of hemodilution on ischemia. In addition, these results suggest that hemodilution, as achieved with DCLHb, was more effective in reducing ischemic brain damage than was the same degree of hemodilution as achieved with albumin.

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DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb™): CONTROL OF PRESSOR EFFECT WITH ANTI-HYPERTENSIVE AGENTS.

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ABSTRACT

Diaspirin crosslinked hemoglobin (DCLHb™) administration elevates mean arterial pressure (MAP). The purpose of this study was to determine whether commonly used antihypertensive agents could control this pressor effect in rats. Awake rats were injected intravenously (iv) with 280 mg/kg of DCLHb. Fifteen minutes later when MAP was 25-30% above baseline and heart rate (HR) was reciprocally decreased, prazosin (2 mg/kg;an alpha adrenergic blocker), nitroglycerine (NTG; 10-150 mcg/min; a nitrovasodilator), nicardipine (0.204-0.08 mg/hr; a calcium channel blocker) or labetalol (5 mg/kg; an alpha/beta adrenergic blocker) was administered iv. All four classes of antihypertensive agents promptly restored MAP to baseline. Coincident with the return of MAP to baseline, HR was restored to baseline in prazosin and NTG treated animals, however, bradycardia persisted in those animals treated with nicardipine and labetalol, most likely due to the negative chronotropic properties of these agents. We conclude that the pressor effect of DCLHb can be readily controlled with at least four different classes of commonly used antihypertensive agents.

INTRODUCTION

Diaspirin crosslinked hemoglobin (DCLHb), a chemically stabilized stroma-free hemoglobin solution, holds promise as an oxygen-carrying fluid

819

because of its excellent oxygen-transport properties [1], biochemical stability [1] and proven efficacy as a resuscitative fluid in animal models of shock [2]. We have previously reported that DCLHb infusion is associated with a significant increase in MAP which does not adversely affect perfusion [3]. Intravenous administration of DCLHb produces a consistent 25-30% increase in MAP over a wide range of doses (125-4000 mg/kg) while the duration of the pressor response appears to be dose-related [4]. The pressor effect of DCLHb may be advantageous when used as a resuscitative fluid, especially in the young and otherwise healthy trauma patient. However, in certain clinical situations the pressor response to DCLHb may not be desired.

Since DCLHb is being considered for clinical use, we determined whether commonly used antihypertensive agents could control the pressor response of DCLHb when given at clinically relevant doses. Four antihypertensive agents were chosen including an alpha-adrengeric blocker (prazosin), a mixed alpha and beta adrenergic receptor blocker (labetalol), a calcium channel blocker (nicardipine) and a nitrovasodilator (nitroglycerin).

MATERIALS AND METHODS

Conscious rats (250-350 g) with indwelling venous (for infusions) and arterial (for cardiovascular monitoring) catheters were injected iv with a 280 mg/kg bolus of DCLHb (Baxter Healthcare, Round Lake, IL). This dose of DCLHb has been shown to consistently produce a 30-35% increase in resting MAP that peaks between 15-25 minutes and returns to baseline by 120 minutes. At 15 minutes post-DCLHb, one of the following antihypertensive agents was administered iv: prazosin (2 mg/kg, over 1 min), nitroglycerin (NTG; 10-150 mcg/min, to effect), nicardipine (0.204-0.080 mg/hr, 20 min infusion) or labetalol (5 mg/kg, bolus). MAP and heart rate (HR) were continuously monitored for up to 120 minutes post-DCLHb administration. The number of animals in each treatment group was 6-8.

DCLHb was diluted to 7% or 10% protein with balanced electrolyte and buffer solution during preparation. Prior to infusion, DCLHb was filtered

through a 20 micron pore size microaggregate filter and warmed to 37°C. Methemoglobin concentration was <8% at the time of infusion.

Statistical significance ($p \leq 0.05$) between treatment groups was determined using ANOVA with Duncan's Multiple Range test or the paired t-test and is noted with an asterisk in the figures.

RESULTS

Bolus injection of 280 mg/kg DCLHb elicited a prompt increase in MAP which peaked at 15-25 minutes (133 ± 4 mm Hg at 15 min; $p \leq 0.05$) and returned to baseline (105 ± 2 mm Hg; $p \leq 0.05$) by 120 minutes. HR decreased in response to the increase in MAP and returned to baseline with MAP.

A single bolus injection of prazosin, 15 minutes post-DCLHb injection, quickly reduced MAP to baseline and mantained MAP at baseline for up to 2 hrs (Fig. 1). HR was also restored to baseline after prazosin treatment.

Following infusion of NTG, MAP fell steadily from a peak of 141 ± 7 mm Hg at 15 min post-DCLHb to 113 ± 5 mm Hg at 25 min post-DCLHb (Fig. 2) After discontinuation of NTG infusion, MAP was still significantly reduced compared to untreated animals. HR was restored to baseline by 15 minutes post-infusion and remained at or above baseline for the remainder of the experiment.

Nicardipine immediately reduced MAP to baseline and maintained it there for the duration of the infusion (Fig 3). Upon discontinuation of the nicardipine infusion, MAP rebounded but still remained significantly lower than the MAP in untreated animals. Bradycardia persisted in nicardipine-treated animals despite a return of MAP to baseline. Statistically there was no difference between HR in treated and non-treated animals.

Labetalol reduced MAP to baseline within 5 minutes of injection and maintained MAP at baseline for at least 120 minutes (Fig 4). Bradycardia persisted to the same degree in treated and untreated animals for 2 hrs.

DISCUSSION

The observation that alpha-adrenergic receptor blockade, calcium channel blockade and the administration of a nitric oxide donor all reverse
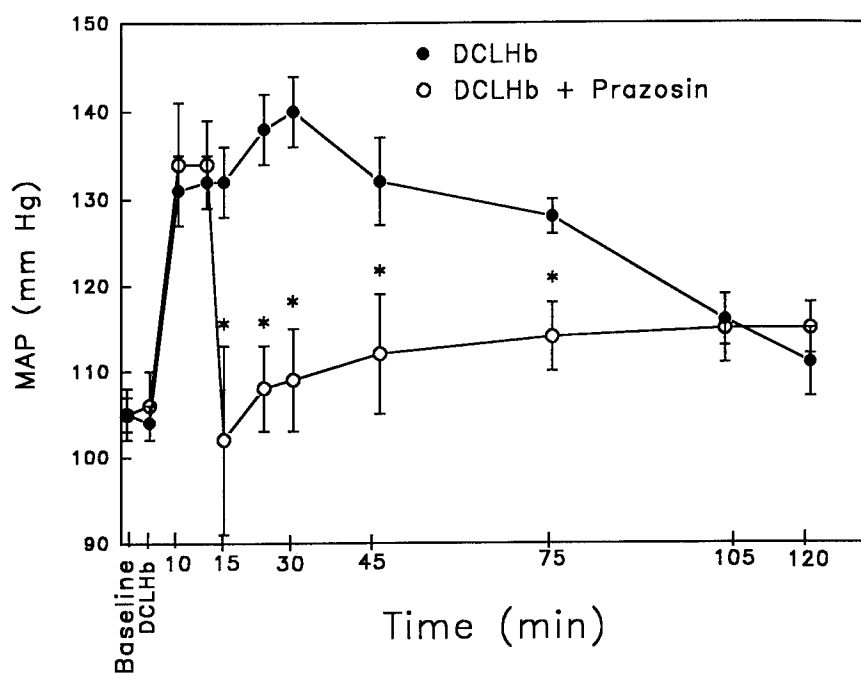
FIGURE 1: Effects of Prazosin (2 mg/kg, iv) on DCLHb-induced mean arterial pressure (MAP) response.

the pressor response to DCLHb suggest that catecholamines, nitric oxide and calcium all play a role in the etiology of this response in rats. These findings are consistent with reports that unmodified oxyhemoglobin elevates blood pressure through the binding of nitric oxide [5] and through calcium mediated vascular smooth muscle contraction [6]. This is the first report implicating adrenergic involvement in the pressor response to DCLHb.

In addition to neurohumoral control of MAP, vascular smooth muscle tone is known to be controlled by the release of vasoactive substances from endothelial cells. Nitric oxide, a potent vasodilator, is released by endothelial cells to counter the effects of endothelin, another endothelial-derived
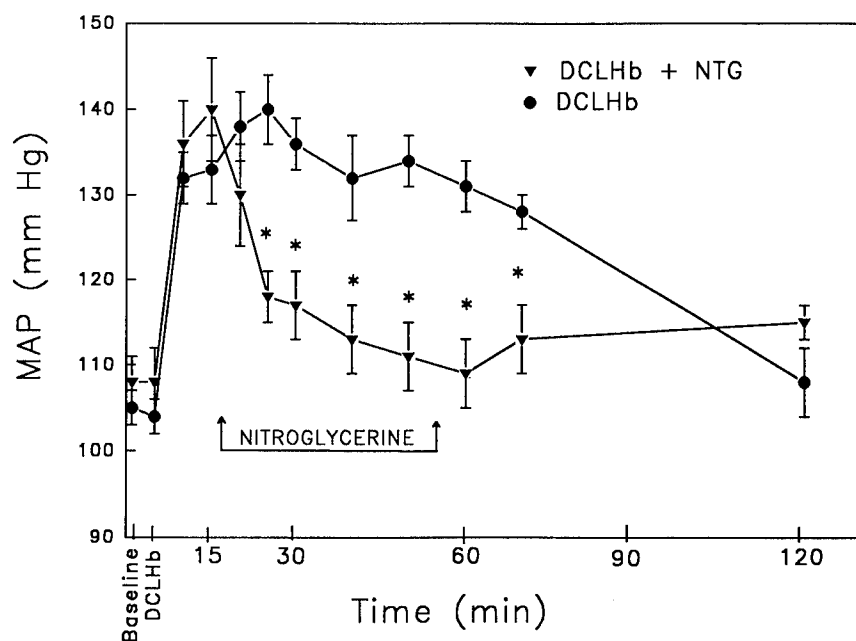
FIGURE 2: Effects of nitroglycerin (NTG) infusion (10-150 mcg/min) on DCLHb-induced mean arterial pressure (MAP) response.

vasoactive substance. The inhibition of nitric oxide can result in an increase in vascular tone and MAP. Since NTG provides an exogenous source for nitric oxide, the observation that NTG reverses the pressor response of DCLHb suggests that DCLHb interacts with nitric oxide. In support of this hypothesis, other studies in rats have shown that L-arginine, a substrate for nitric oxide synthesis, reverses the pressor response to DCLHb [7]. Furthermore, cyanometDCLHb, a modified form of DCLHb that is incapable of binding NO, does not increase MAP upon infusion into rats [7].

Calcium channel blockers are known to decrease tone in vascular smooth muscle by inhibiting calcium entry through voltage gated channels. Since the final common pathway for vascular smooth muscle contraction is
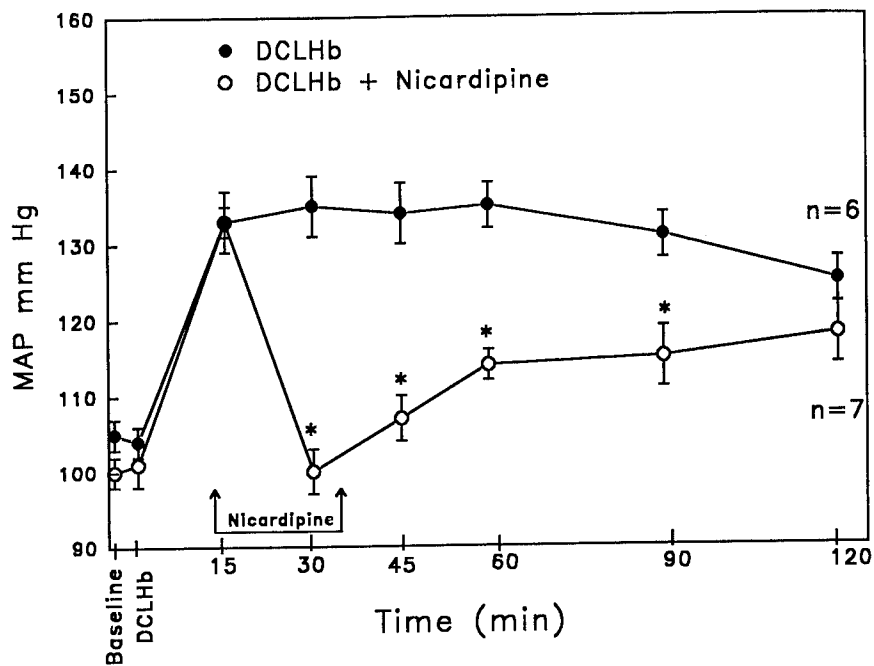
FIGURE 3: Effects of nicardipine infusion (0.204-0.08 mg/hr) on DCLHb-induced mean arterial pressure (MAP) response.
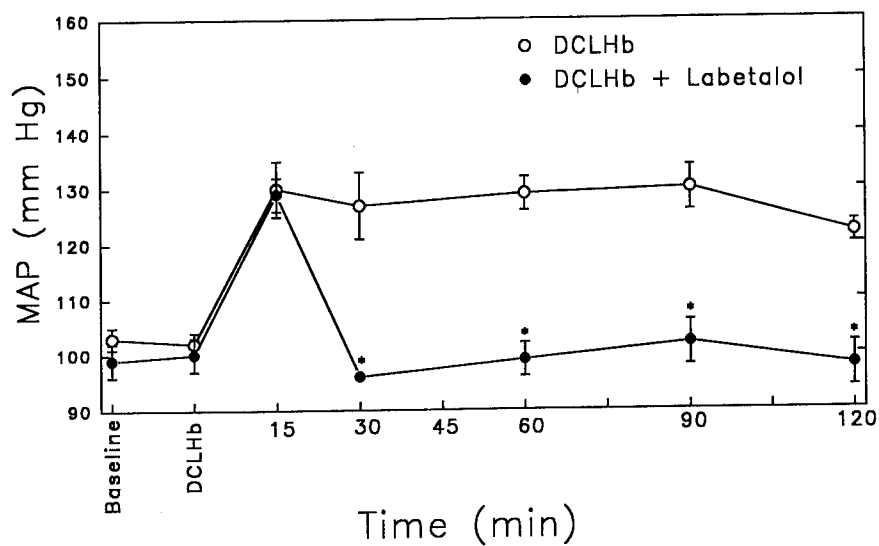
FIGURE 4: Effects of labetalol (5 mg/kg, iv) on DCLHb-induced mean arterial pressure (MAP) response.

intracellular calcium entry, it is not surprising that nicardipine reversed the pressor effect of DCLHb. The persistence of bradycardia seen once MAP was normalized may be due to a negative chronotropic effect of calcium channel blockade.

While the pressor response to DCLHb has not been shown to adversely affect tissue perfusion or oxygen delivery [3], an increase in blood pressure may not be well tolerated or desirable under certain clinical situations. To this end we have shown that the pressor response to DCLHb can be readily controlled with at least four commonly used anti-hypertensive agents. Furthermore, the doses of the antihypertensive agents used in this study approximate the doses used clinically to treat hypertension. Finally, these findings have provided insights into the mechanism(s) involved in the etiology of this pressor response.

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OXYGENATION OF THE RAT 9L GLIOSARCOMA AND THE RAT 13672 MAMMARY CARCINOMA WITH VARIOUS DOSES OF A HEMOGLOBIN SOLUTION

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ABSTRACT

Tumor oxygen tensions were measured using a computer controlled pO_2 microelectrode in two preclinical solid tumor models, the rat 9L gliosarcoma and the rat 13672 mammary carcinoma. Tumor oxygenation profiles were determined under four conditions: 1) normal air breathing, 2) carbogen (95% O_2/5% CO_2) breathing, 3) after intravenous administration of a solution of ultrapurified polymerized bovine hemoglobin with normal air breathing and 4) after intravenous administration of a solution of ultrapurified polymerized bovine hemoglobin with carbogen breathing. Both tumors had severely hypoxic regions under normal air breathing conditions. Although carbogen breathing increased the oxygenation of the better oxygenated portions of the tumor, it did not impact on the severely hypoxic tumor regions. Administration of the hemoglobin solution was effective in increasing the oxygenation throughout both tumors under normal air breathing conditions. The addition of carbogen breathing to administration of the hemoglobin solution eliminated severe hypoxia in the 9L gliosarcoma and markedly reduced the severely hypoxic regions of the 13672 mammary carcinoma.

INTRODUCTION

Cancer patients are usually anemic and evidence is accumulating that anemia is an important prognostic factor. Furthermore, increasing hemoglobin levels into the normal range can improve prognosis and treatment outcome [1, 2]. Solid tumor masses are very heterogeneous in oxygenation and contain regions of hypoxia [1-4]. Preclinical studies both in vitro and in vivo have established that hypoxia protects cells from the cytotoxic actions of radiation and many chemotherapeutic agents and thereby may be a significant factor in therapeutic resistance [5-7].

An ultrapurified polymerized bovine hemoglobin solution administered i.v. to C3H mice bearing the FSaIIC fibrosarcoma has been shown to increase both the tumor growth delay and tumor cell killing by radiation therapy and by a variety of chemotherapeutic agents with little increase in the toxicity of these agents to the bone marrow [8, 9]. In the current report, we describe the effect of the same ultrapurified bovine hemoglobin solution on the oxygenation of the rat 9L gliosarcoma and the rat 13672 mammary carcinoma.

MATERIALS AND METHODS

Drug. The hemoglobin solution (Biopure Corp., Boston, MA) is a polymerized form of a highly purified bovine hemoglobin solution that contains 13 ± 2 gm/dl bovine hemoglobin. P_{50} measurements of the hemoglobin solution under conditions designed for testing human hemoglobin gave values of 34-37 mmHg. The hemoglobin component has a molecular mass range from 64 kDa to 500 kDa (w/v). The hemoglobin solution also contains sodium (150 mM), chloride (110 mM), and potassium (4.0 mM) in a buffer solution (pH 7.6-7.9). The circulating half-life of the preparation is about 2 days [10, 11]. Carbogen is 95% oxygen and 5% carbon dioxide.

Tumors. Rat 9L Gliosarcoma. 9L gliosarcoma cells were obtained as a gift from Dr. Dennis Deen (University of California, San Francisco, CA). This tumor is carried in the female Fischer 344 rat. The tumor grows to 100 mm^3

in about 12 days when implanted s.c. in the legs of female rats [12].

Rat 13672 Mammary Adenocarcinoma. This is a carcinogen induced (DMBA) tumor of the female Fischer 344 rat. The tumor can metastasize to the lungs and abdominal organs. The tumor is composed of epithelial tissue in folds and acini. The tumor grows to 100 mm^3 in about 14 days when implanted s.c. in the legs of female rats.

Oxygen Measurements. Tissue oxygen measurements were made using a pO2-Histograph (Eppendorf, Inc., Hamburg, Germany). The polarographic needle microelectrode was calibrated in aqueous solutions saturated with air and 100% nitrogen. The electrode was used for tumor measurements if there was less than 1% variation in current measurements upon repetition of the calibration cycle. For tumor pO$_2$ measurements, the animal was anesthetized by an i.p. injection of Ketaset (35 mg/kg) and xylazine (25 mg/kg) prepared in phosphate-buffered 0.9% saline. The animal was placed on a heating pad and covered with a blanket to maintain body temperature. Core temperature was measured with a rectal thermometer. The tumor site was shaved and tumor diameters measured with calipers. A small patch of skin about 2 cm from tumor was shaved and a small incision was made allowing the reference electrode (Ag/AgCl-ECG) to be inserted subcutaneously and secured. The tumor was exposed by removing about 0.5 cm^2 of skin over the site. The tumor capsis was perforated with a 20-gauge needle. The pO$_2$ microelectrode was positioned in the perforation.

The pO$_2$ microelectrode under computer control enters 1 mm into the tissue and then retracts 0.3 mm. Probe current is then measured and after 1.4 seconds the probe moves forward again. The total length to the measurement path is determined by the size of the tumor. After the probe reaches the end of its measurement path it automatically retracts. The probe was then repositioned in the same perforation at a different angle and stepwise measurements again initiated. Three diameters were measured in each tumor for a total of 50-60 measurements per condition.

Tumor pO_2 measurements were made under four conditions: 1) normal air breathing, 2) carbogen (95% O_2/5% CO_2) breathing, 3) 10 minutes post intravenous hemoglobin solution administration with normal air breathing and 4) 15 minutes post the initiation of carbogen breathing after intravenous hemoglobin solution administration.

Each tumor-bearing rat underwent tumor pO_2 measurements under four experimental conditions, therefore each tumor was probed four times through three diameters. Data collection through three tumor diameters accrued about 50 pO_2 measurements and took about 10 minutes. The pO_2 microelectrode was recalibrated in aqueous solutions saturated with air and 100% nitrogen after each data collection, therefore the pO_2 microelectrode was recalibrated 4 times during the course of the experiment. Recalibration requires about 15 minutes. Therefore, the duration required for tumor pO_2 measurements under the four conditions tested was about one hour and 40 minutes.

RESULTS

TABLE 1 shows several parameters related to the oxygenation of the rat 9L and 13672 tumors. Under normal air breathing conditions almost half pO_2 measurements in the 9L tumors <5 mmHg which indicates severe hypoxia. Under this same condition the mean and median pO_2 of the 9L tumors is similar indicating that the pO_2 distribution in the tumor is relatively normal. Carbogen breathing increased the mean pO_2 in the tumor more than the median pO_2, thus carbogen breathing provides more oxygen primarily to those tumor regions which were better oxygenated (near to the vasculature of the tumor). Administration of the hemoglobin solution to rats bearing the 9L tumor improved the oxygenation throughout the tumor as reflected by relatively equal increases in the mean and median pO_2 and substantially reduced the severely hypoxic regions of the tumor. The addition of carbogen breathing to treatment with the hemoglobin solution eliminated hypoxia in the 9L tumor reducing the percent of pO_2 readings of <5 mmHg to 0.

TABLE 1.Oxygenation Parameters for the Rat Tumors Under Several   Conditions

Measurement condition	Mean/Median pO2, mmHg	% of pO2 readings <5 mmHg	pO2, mmHg percentiles 10th	90th
9L GLIOSARCOMA				
air	7.4/6.5	49	0.0	28
carbogen	42.2/25.0	41	0.0	116
Hemoglobin Solution (12 ml/kg)				
air	17.6/18.2	24	2.7	29
carbogen	68.8/55.4	0	14.5	152
13672 MAMMARY CARCINOMA				
air	11.3/4.9	52	0.0	34
carbogen	31.6/15.1	42	0.0	81
Hemoglobin Solution (4ml/kg)				
air	13.5/4.9	52	0.0	44
carbogen	21.4/14.7	39	0.2	49
Hemoglobin Solution (8 ml/kg)				
air	20.8/16.6	44	0.8	51
carbogen	37.6/25.3	29	1.2	86
Hemoglobin Solution (12 ml/kg)				
air	21.6/17.0	40	0.0	75
carbogen	50.2/39.6	0	8.6	98

The effect of a range of hemoglobin solution doses on tumor oxygenation was studied in the rat 13672 mammary carcinoma. Under normal air breathing conditions 52% of the 13672 tumor had oxygen levels < 5 mmHg. Carbogen breathing increased both the mean and median pO2 of the 13672 tumor and reduced the regions of severe hypoxia to 42% of the tumor. Administration of 4 ml/kg of the hemoglobin soution did not significantly alter the oxygenation of the 13672 tumor under air breathing or carbogen breathing conditions. Administration of 8 ml/kg of the hemoglobin solution increased the mean and median

pO2's of the tumor and reduced the severely hypoxic re-
gions of the tumor to 44% under air breathing.
Administration of a higher dose of 12 ml/kg of the
hemoglobin produced the same effect on tumor oxygena-
tion under air breathing conditions as did the 8 ml/kg
dose. Carbogen breathing further increased the oxygen
tensions in the tumor so that at a dose of 8 ml/kg of the
hemoglobin solution the severely hypoxic regions were re-
duced to 29% of the tumor and with a dose of 12 ml/kg of
the hemoglobin solution severe hypoxia was eliminated
from the tumor.

DISCUSSION

The ability to measure tissue oxygen pressure in a
stable, well-controlled manner is a recent development [2].
Regions of hypoxia which could be considered therapeuti-
cally significant (5 mmHg or less) comprised a substantial
portion of both of the tumors in this study. In solid tumor
model systems hypoxic cells have been shown to limit the
response of neoplasms to treatment with ionizing radiation
as well as to many chemotherapeutic agents [6, 7].

The preparation of polymerized bovine hemoglobin
used in this study was manufactured by an improved pu-
rification process, has undergone extensive preclinical
testing and appears to have successfully overcome the
limitations of previous hemoglobin preparations [10, 11,
13]. Most significantly, administration of moderate doses
of the hemoglobin solution were effective in increasing the
oxygenation in both the 9L and 13672 tumors under nor-
mal air breathing conditions which is a very positive factor
in the potential clinical application of this material.

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MEASUREMENT OF INTERLEUKIN-6 PRODUCTION BY MONOCYTES FOR IN VITRO SAFETY TESTING OF HEMOGLOBIN SOLUTIONS

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ABSTRACT

Induction of interleukin-6 (IL-6) production by isolated human mononuclear blood cells was taken as in vitro model for the induction of inflammatory reactions. The model was very sensitive to bacterial endotoxin (detection limit < 10pg/ml). Hemoglobin (Hb) solutions, prepared under non-sterile conditions also induced IL-6 production, which correlated with a positive reaction in the Limulus assay. Purification of the Hb solutions with a detergent prevented IL-6 production, showing that pure Hb itself does not activate the monocytes. We conclude that this assay is a useful and sensitive test of contamination with components that can induce inflammatory reactions, especially microbial products.

INTRODUCTION

The main barrier to clinical application of Hb solutions as blood substitutes are the potential toxic effects. In the past 20 years several toxic factors have been recognized, such as the role of erythrocytic stroma in causing coagulation abnormalities. However, some adverse reactions of varying severity, observed in limited clinical trials, remain unexplained. Therefore, we are conducting an extensive safety study of our Hb solution, including in vivo and in vitro testing. Because the Mononuclear Phagocyte System (MPS) plays a primary role in inflammatory reactions, we evaluated the activation of human monocytes as an in

vitro toxicity assay. Other investigators have found that certain Hb solutions may activate several monocyte functions (1,2). In the present study we measured the production of Interleukin-6 (IL-6), as it is the major mediator in the acute phase reaction and fever response.

MATERIALS AND METHODS

Polymerized Hb solutions were prepared from human blood essentially as described before (3). Mononuclear cells (15% monocytes) were isolated from the buffy coat of a unit of human donor blood by centrifugation on a Ficoll (1.078 g/ml) layer, and were stored in 30 - 60 portions at -180°C, using DMSO as cryoprotectant. After thawing and washing, the mononuclear cells were incubated overnight at 37°C in Iscove's modified Dulbecco's medium (IMDM) + 5% fetal calf serum in 96-well microtiter plates (60000 cells in 200μl per well). Hb solutions or other test samples were added in a 1:256 to 1:4 ratio to the culture medium. Alternatively, incubations were done with whole, heparinized blood (diluted 1:10 with IMDM) instead of isolated mononuclear cells. The IL-6 concentrations in the supernatant were measured with an ELISA as described by Helle et al (4) (detection limit: 5pg IL-6 per ml).

Several samples of Hb were purified by phase separation with the detergent Triton X-114 (Sigma) at 4°C as described by Aida and Pabst (5). Triton was removed by centrifugation at 37°C and subsequent addition of adsorbents (Bio-Beads SM, Bio-Rad). E.coli O55:B5 endotoxin was obtained from Sigma (1ng $\approx$ 10EU). Endotoxin concentrations were measured with a chromogenic Limulus assay (Coatest, KabiVitrum, Sweden).

RESULTS

First, we tested the sensitivity of the assay to bacterial endotoxin. There were differences between different batches of mononuclear cells in background and maximum IL-6 production (see figure 1). However, the detection limit for E. coli endotoxin was about the same in all experiments: less than 10 pg/ml.

Thereafter, we tested sixteen batches of Hb solutions that had <u>not</u> been prepared under aseptic conditions, with both the monocyte assay and the limulus assay. All batches had been sterilized by 0.22μm filtration at the and of the production process. Figure 2A shows that ten of these batches induced a high IL-6 production
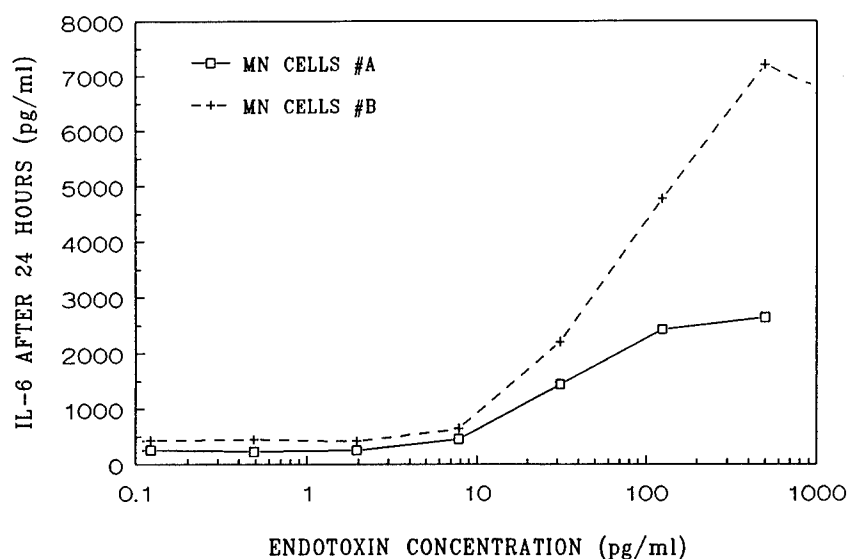
FIGURE 1   Dose-response curves for E. coli endotoxin, showing the results with two
different batches of mononuclear cells

in a dose-dependent manner (Hb concentration: 0.025 - 1.6 g/100ml). Batch #4
inhibited IL-6 production at the higher concentrations, indication the presence of
inhibiting factor. The same batches were also positive in the Limulus assay (figure
2B).

Four of the positive samples were pretreated with Triton X-114. This procedure
effectively removed contaminating endotoxin as measured with the limulus assay.
It also completely eliminated the IL-6 inducing capacity of the Hb solutions.
Control experiments in which the pretreated Hb samples were spiked with
endotoxin, showed that the Triton purification left no toxic substances that
inhibited the monocytes.

In another series of experiments we examined the effect of contaminating
erythrocytic stroma. We found that sonicated erythrocytes gave no stimulation of
the monocytes, but, on the contrary, inhibited the background IL-6 production.
Moreover, addition of small quantities of erythrocyte lysate (Hb conc 0.025g%)
shifted the dose-response curve for endotoxin to the right by a factor of two,
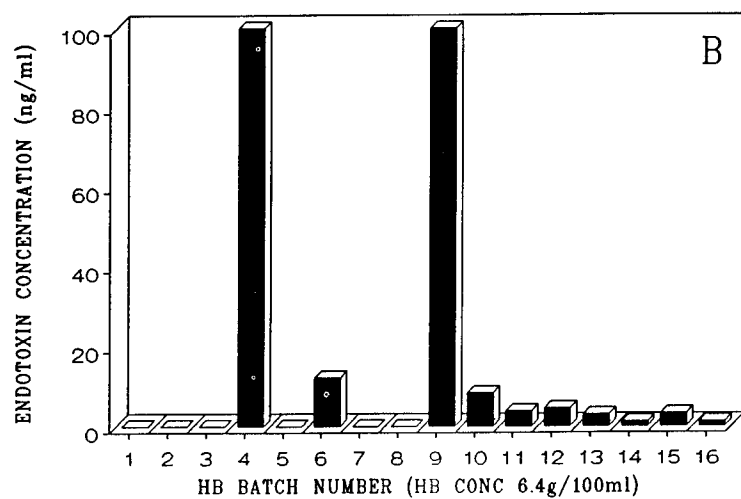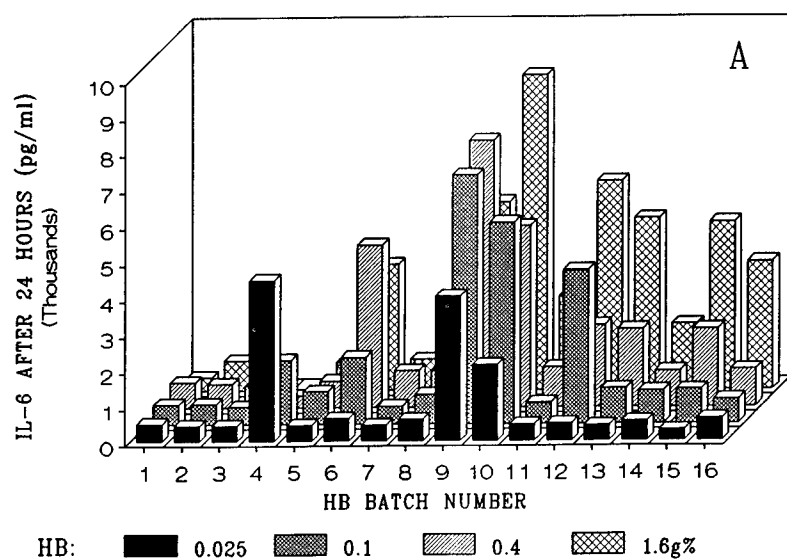
FIGURE 2 Panel A shows induction of IL-6 production by 16 batches of Hb solutions
(final Hb concentration 0.025 - 1.6 g/100ml) that had not been prepared under aseptic
conditions. Panel B shows the endotoxin concentrations that were measured in the same
batches using the limulus assay.

without decreasing the maximum IL-6 concentration, indicating a competitive inhibition.

Similar experiments were performed by incubating whole blood instead of isolated mononuclear cells. This procedure gave almost undetectable background IL-6 production, while the detection limit for endotoxin was still below 10 pg/ml. Preliminary experiments showed that the effects of contaminated Hb solutions were about the same.

DISCUSSION

Our experiments revealed that pure polymerized Hb itself neither induces nor inhibits IL-6 production by human mononuclear blood cells in vitro. Hb batches that had not been prepared under aseptic conditions, strongly induced IL-6 release. The fact that this correlated with a positive reaction in the Limulus assay suggests involvement of contaminating endotoxin. This is supported by the prevention of IL-6 release after pretreatment of the Hb solutions with Triton X-114, a procedure which effectively removes endotoxin from protein solutions. However, because the purification with Triton X-114 is not specific for endotoxin it is possible that also other contaminants in the Hb solutions were implicated in the activation of the IL-6 production. We can rule out contaminating stroma because sonicated erythrocytes did not activate the IL-6 production. But, other microbial components than endotoxin, like components from Gram positive bacteria, might be involved because they can also induce IL-6 production in monocytes.

We conclude that the measurement of IL-6 release by monocytes in vitro provides a very sensitive assay for testing Hb solutions on contamination with products that can induce inflammatory reactions, especially bacterial products. The assay has a detection limit for endotoxin in Hb solutions below 40 pg/ml (0.4 EU/ml) when the Hb sample is added in a 1:4 ratio to the culture medium and is in this respect more sensitive than the rabbit pyrogen test. Moreover, the monocyte assay has the advantage over the rabbit pyrogen test that it is suitable for testing large numbers of samples and thus also for in-process control. The monocyte assay has the advantage over the Limulus assay that the sensitivity is not limited to contamination with endotoxin.

On comparing the mononuclear cell culture with whole blood incubation, we consider the latter to be the method of choice because it more closely mimics the

in vivo conditions and avoids manipulating of the monocytes. To overcome the problem of differences in sensitivity between individual donors we standardized the assay by comparing the induction IL-6 production by hemoglobin samples with a dose-response curve of a reference endotoxin.

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FUNCTIONAL CAPILLARY DENSITY CHANGES DURING BLOOD SUBSTITUTION WITH ααHb and DEXTRAN 70: INFLUENCE ON OXYGEN DELIVERY

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ABSTRACT

The effectiveness of a blood substitute is ultimately determined by the rate at which O_2 arrives to the capillaries and the functional capillary density i.e., the number of flowing capillaries per unit volume of tissue. We use this rationale to analyze the effectiveness of isovolemic blood substitution with ααHb (3,5-bis(dibromosalicyl)fumarate) compared to isooncotic and isovolemic hemodilution with dextran 70.

Progressive hemodilution with each solution was performed in the awake hamster skinfold model by simultaneous isovolemic exchange of blood until the systemic hematocrit was reduced to 30% of control. Systemic hematocrit, blood pressure, heart rate were monitored. To determine O_2 delivery at the microcirculatory level, functional capillary density, RBC velocity, RBC flux, capillary hematocrit were measured.

Functional capillary density was maintained during moderate hemodilution with dextran 70, whereas ααHb exchange caused a gradual reduction in the number of flowing capillaries. O_2 delivery to tissue was calculated from total O_2 content (RBC and plasma Hb or RBC only), blood

flow, and functional capillary density. Our findings suggest that augmentation of the O_2 content of blood with $\alpha\alpha$Hb substitution produces similar results in terms of capillary O_2 delivery and capacity as isovolemic and isooncotic hemodilution with dextran 70.

INTRODUCTION

Hemodilution, a reduction in number of circulating RBCs, results in lower oxygen carrying capacity of blood. Blood substitution with dextran 70 changes rheological properties of blood, thereby altering microvascular hemodynamics. The reduction in blood viscosity resulted in 1) an increase in RBC velocity in the capillaries, 2) maintenance of RBC flux, and 3) a nonproportional relationship between the drop in systemic and capillary hematocrit [4]. Moderate hemodilution with dextran 70 increases the oxygen transport capacity of blood, although it is a volume replacement fluid without oxygen carrying capabilities [3]. It is hypothesized that a blood substitution with an oxygen carrying substance would augment the oxygen carrying capacity of blood and thus present the tissue with more oxygen than would have occurred with a simple hemodilution with a colloid or crystalloid fluid. Study of changes in mixed arteriolar-venous pO_2 from organs may provide only partial information regarding actual levels of tissue oxygenation as events such as tissue perfusion occur at the microscopic level and are dynamic. Direct studies of the microcirculation at the level of the capillaries may provide additional information on how oxygen is delivered to tissue.

The objective of our study is to characterize the levels of tissue perfusion and oxygen delivery to tissue during isovolemic substitution with $\alpha\alpha$Hb as compared to dextran 70.

MATERIAL AND METHODS

Animal Preparation

Studies were carried out in the hamster skin fold model, which is described fully elsewhere [1,5]. This model allows for the direct observation

of the intact subcutaneous microvasculature of an awake, unanesthetized animal. Chamber implantation and catheterization of the right jugular vein and left carotid artery were performed at least two days prior to the investigation, thus mitigating any possible complications from post-surgical trauma.

Blood Substitution

Progressive levels of blood substitution was performed by simultaneous withdrawal of blood from the arterial catheter and infusion of the test solution into the venous catheter at a rate of 0.1 ml/min. Two test solutions were used: dextran 70 and $\alpha\alpha$Hb. The test solution was warmed to 39 degree centigrade by immersion in a controlled water bath. Prior to infusion into the animal, the $\alpha\alpha$Hb solution was passed through a 0.22 μm syringe filter as a safeguard against possible administration of aggregates. The first blood substitution was an exchange of 1.5 ml; all subsequent exchanges were 1.0 ml. The animal was given a 5 minute recovery period to stabilize prior to the recording of the microscopic fields under study. Heart rate and blood pressure were monitored and systemic hematocrit measured. This protocol was continued until systemic hematocrit was reduced to 30% of control.

Experimental Procedure

Systemic and microcirculatory control parameters were assessed prior to blood substitution. The baseline blood pressure was measured from the arterial catheter and heart rate was estimated from the tracing. Systemic hematocrit was measured from centrifuged arterial blood samples. Systemic and microhemodynamic parameters were again assessed after each blood substitution.

Functional capillary density was obtained by sequentially observing a region comprised of 5 horizontal by 2 vertical microscopic fields (total area = 0.05 mm^2), for 2 minutes. A capillary is deemed functional if a RBC transverses the vessel during the observation period.

Oxygen Transport Capacity

Oxygen Transport Capacity was calculated from the expression:

Oxygen transport capacity =
        RBC Flux x Functional Capillary Density x Oxygen Content.

The oxygen content for the $\alpha\alpha$Hb is a function of the capillary RBC hematocrit and the concentration of cell free hemoglobin in the plasma. For dextran 70, the oxygen content is solely a function of the capillary RBC hematocrit.

RESULTS

Three blood exchanges following the above protocol were needed to reduce the systemic hematocrit to at least 30% of control. The animals exhibited a slight increase in mean arterial pressure upon the first exchange with $\alpha\alpha$Hb. A slight decrease in pressure was recorded by the third blood exchange with both solutions. These trends however were not statistically significant. There was also no statistically significant changes in heart rate during the experiment.

The RBC flux was maintained relative to control for the first exchange, however it was reduced upon further blood dilution. Increased RBC velocity was observed until the second blood exchange at which point the velocity decreased relative to control. The drop in capillary hematocrit was not proportional to the reduction in systemic hematocrit.

The baseline level of functional capillary density was maintained during exchange with dextran 70 until the last blood exchange. $\alpha\alpha$Hb exchange resulted in a immediate 30% decrease in functional capillary density which reached a maximum of 48% by the last exchange.

Figure 1 compares the oxygen transport capacity achieved with $\alpha\alpha$Hb and dextran 70. The addition of an oxygen carrying hemoglobin did not significantly elevate oxygen transport capacity.
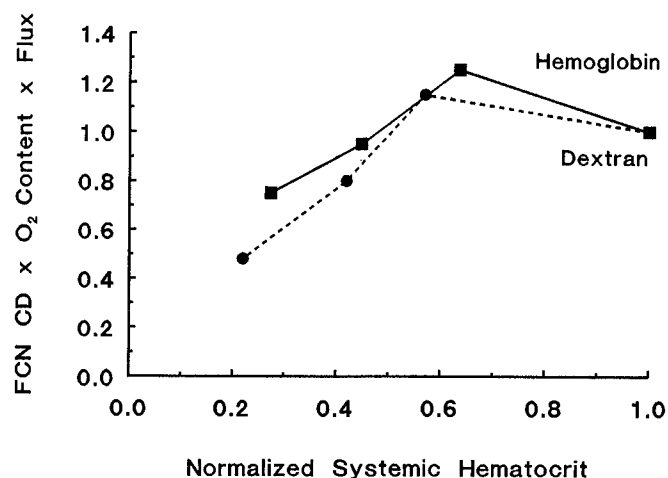
FIGURE 1. Oxygen Transport during Isovolemic Blood Substitution. Plot of normalized systemic hematocrit as a function of the oxygen carrying capacity of blood during different levels of blood substitution with $\alpha\alpha$Hb (n=9) and dextran 70 (n=5). Similar levels of oxygen transported to tissue are achieved with $\alpha\alpha$Hb and dextran 70 isovolemic exchange.

DISCUSSION

The calculations of oxygen transport capacity are based on the assumption that the $\alpha\alpha$Hb is fully loaded with oxygen, to the same degree as the RBCs. Therefore these calculations may overestimate the actual capacity.

Although the microvascular hemodynamic alterations from the first level of $\alpha\alpha$Hb exchange were similar to those achieved with dextran 70 exchange, the decrease in functional capillary density counteracts the increase in oxygen content of blood relative to dextran 70 thus resulting in a similar level of oxygen transport capacity.

The observed decrease in functional capillary density may or may not be have a detrimental effect on the tissue since the oxygen delivery rate to the tissue is the same as dextran 70. Moderate hemodilution with dextran 70 is a commonly used and successful clinical therapy [3]. A plausible mechanism

for reduced number of flowing capillaries is endothelial swelling which would result in the narrowing of the capillaries, dramatically increasing their hydraulic resistance to the point of no capillary flow [2]. Another hypothesis is that the oxygen level in the tissue is sufficient with a lower functional capillary density since blood is augmented with the addition of a supplementary oxygen carrier resulting in a oxygen carrying capacity similar that of dextran 70.

SUMMARY

The combined changes in microvascular hemodynamics and functional capillary density suggest that augmentation of the O_2 content of blood with $\alpha\alpha$Hb substitution results in similar levels of oxygen delivery as isovolemic hemodilution with dextran 70.

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ENCAPSULATION OF HEMOGLOBIN IN NON-PHOSPHOLIPID VESICLES

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ABSTRACT

The efficiency of encapsulating hemoglobin in non-phospholipid liposomes by rapidly mixing hemoglobin with lipids heated above their solid-liquid phase transition temperature was examined. Human hemoglobin was mixed at 55-60°C with a lipid solution containing polyoxyethylene-2 cetyl ether and cholesterol (molar ratio, 3:1) at 60-65°C. Repeated mixing was carried out through a high-shear orifice, followed by rapid cooling and additional mixing. Lipid vesicles were heterogeneous in size, with diameters from ~300 nm to 10 μm. The non-encapsulated aqueous phase was removed by centrifugation, and total hemoglobin was determined spectrophotometrically. Encapsulation efficiency was calculated as the percentage of hemoglobin associated with the liposome phase (*i.e.*, encapsulated) as a function of hemoglobin concentration and the aqueous:lipid hydration ratio. Hemoglobin concentrations were varied from 1 to 10 mM (in heme). Aqueous:lipid ratios of 8:1 and 4:1 were tested. Percent encapsulation varied from 13-30%, with the greatest efficiency, *i.e.*, 30%, at a 4:1 hydration ratio of hemoglobin:lipid at 5.6 mM hemoglobin.

INTRODUCTION

Red blood cells maintain sufficient oxygen-carrying capacity *in vivo* because of their high concentrations of intracellular hemoglobin. One approach in the design of red blood cell substitutes is to achieve efficient encapsulation of high concentrations of hemoglobin in artificial membrane systems, or liposomes.

Biological membranes, including red blood cell membranes, are primarily phospholipid bilayers, combined with other membrane components. One critical component, the sterol cholesterol, increases membrane stability and fluidity. Thus, phospholipids and cholesterol are now the principal lipids used in liposome production. A common method for manufacturing liposome-encapsulated hemoglobin requires dissolving lipids, which are water insoluble, in an organic solvent, evaporating the solvent to leave a thin film of lipid, and then hydrating the dried lipids in a hemoglobin solution. The resulting multilamellar liposome suspension can be extruded through porous membrane filters to produce homogeneous unilamellar bilayer vesicles [1].

Large-scale processing and sterilization techniques in the manufacture of liposome-encapsulated hemoglobin have been problematic. Furthermore, pure phospholipids are expensive, and the presence of free phospholipids has been associated with toxicities of cell-free hemoglobin solutions [2]. A simpler approach to hemoglobin encapsulation, with a choice between either phospholipid or non-phospholipid components would be advantageous.

In this paper, we report the efficiency of hemoglobin encapsulation by a technique based on dissolution of lipids through temperature phase transition rather than in organic solvent. Both phospholipids and double or single-tailed non-phospholipid amphiphiles can be used to produce lipid microspheres called Novasomes™ [3]. Here, we discuss hemoglobin encapsulation with a lipid mixture containing a single-tailed non-phospholipid amphiphile, polyoxyethylene-2 cetyl ether, and cholesterol.

MATERIALS AND METHODS

Encapsulation procedure:

Solid polyoxyethylene-2 cetyl ether (POE-2) (ICI Specialty Chemicals) and cholesterol (R.I.T.A., Corp.), at a 3:1 molar ratio, were melted rapidly by heating to ~100°C and then allowed to cool to 60-65°C. Stroma-free human hemoglobin (provided by the Center for Blood Research, Boston, MA) was heated separately and rapidly in a water bath to 50-55°C. The lipid and hemoglobin solutions were then loaded into separate pre-heated syringes to the appropriate volumes for the hydration ratio (*e.g.*, for an 8:1 hydration ratio, 8 ml hemoglobin and 1 ml lipid). The temperature differential between lipid and aqueous phases for mixing was ≤10°C. The two liquid phases were mixed by pushing the lipid solution into the aqueous solution through the high-shear orifice of a 3-way metal stopcock. Mixing was continued for 20 strokes back and forth between the two syringes, followed by rapid cooling under cold water with continued mixing for 16-20 more strokes.

Washing procedure:

Unentrapped hemoglobin was separated from the Novasome layer by centrifugation at 3500 RPM for 10 min in 5% dextran-phosphate buffered saline at pH 7.4. The 5% dextran (87 kDa) imparts slightly greater density to the aqueous phase, giving a distinct separation between aqueous and Novasome layers, with the Novasome layer rising to the top. Washing was repeated until hemoglobin was no longer detected in the wash solutions.

Determination of percent encapsulation:

Percent encapsulation was calculated from measurements of the total starting hemoglobin (in moles) minus the moles of hemoglobin washed free of the Novasome layer, after repeated washing, divided by the total. Hemoglobin concentration in each wash fraction was measured by absorbance at 523 nm (ε_{523} = 7.12 mM^{-1}cm^{-1} [4]). A precise measurement of the volume of each wash fraction was determined by weight and density of the aqueous phase.

Denatured hemoglobin was observed as a gray particulate that precipitated during centrifugation. The amount of precipitated protein was unaccounted for and provides a source of error in the calculations.

Visual examinations:

Preparations were examined by light microscopy (400x) for analysis of homogeneity, vesicle aggregation, size and shape. Vesicle sizes were estimated by subjective comparison with calibrated polystyrene microspheres.

RESULTS

Percent encapsulations are given in **Table I**. Encapsulation efficiency decreased from 21 to 14% by increasing hemoglobin concentration from 1 to 10 mM. Decreasing the hydration ratio increased encapsulation of 10 mM hemoglobin to 22%. The highest percentage of encapsulation (30%) was observed with a 4:1 hydration ratio and 5.6 mM hemoglobin.

Light microscopy revealed heterogeneous vesicles, ranging in size from ~0.3-10 μm in diameter. Because the vesicles are osmotically active, their size and shape depend on the ionic composition of the suspension medium.

DISCUSSION

Encapsulation efficiency was calculated from the total initial hemoglobin minus free hemoglobin in the aqueous phase separated from the Novasome layer by centrifugation divided by the initial total hemoglobin. We have not shown that the hemoglobin associated with the Novasome layer is entirely intravesicular; some of the protein may be bound to the outside layer of the lipid membrane. To try to test for this, we prepared a control for each Novasome formulation: Novasomes made using buffer without hemoglobin were mixed afterwards with the hemoglobin solution in a test tube (*i.e.*, low shear) and subjected to the same washing procedure. Within the errors of our

TABLE I

Percent encapsulation of Hemoglobin

Liposome formulation (Molar ratio)	Hydration ratio (aqueous:lipid)	[Hb] (mM)	% (final after washing)
POE-2/cholesterol (3:1)	8:1	1	21.0 ± 3.0[a]
POE-2/cholesterol (3:1)	8:1	10	13.5 ± 0.5[a]
POE-2/cholesterol (3:1)	4:1	10	22
POE-2/cholesterol (3:1)	4:1	5.6	30

[a]Average of 2 trials ± the range.

calculations, no hemoglobin was found to be associated with these Novasome preparations under any conditions after washing. This may not be conclusive, however, if high-shear mixing is a requisite for hemoglobin association with the outer surface of the lipid bilayer.

Percent encapsulation decreased as hemoglobin concentration was increased and increased as the hydration ratio was decreased. A reduction in the hydration ratio from 8:1 to 4:1 increases the amount of lipid relative to the aqueous phase and thus provides a higher degree of encapsulation. At high concentration, i.e., 10 mM hemoglobin, the lipid/aqueous solution was viscous and manual mixing could have been inadequate. An automated process that supplies consistent hydrodynamic mixing forces might improve encapsulation efficiencies at high hemoglobin concentrations and/or decrease heterogeneity in vesicle size.

The hemoglobin denaturation observed in these experiments probably resulted from heating and/or mechanical shear. The detrimental effect of heating on hemoglobin solutions may be circumvented in the future by using a modified hemoglobin, such as a cross-linked hemoglobin, with improved heat stability [5].

The method described here is fast, simple and inexpensive: (1) it requires unsophisticated techniques of heating and mixing, (2) non-phospholipids can be used, (3) organic solvents are not required, (4) encapsulation efficiencies are reproducible but may vary as a function of hemoglobin concentration or hydration ratio, and (5) viral inactivation potentially may be achieved during the phase-transition heating step.

The experiments described here were conducted on a small scale, *i.e.*, 10 ml, but large-scale production, using the same procedure, should be feasible with appropriate re-engineering of the process.

Author's (KDV) note: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the U.S. Department of the Army or the Department of Defense.

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STRUCTURE AND SOLUTION PROPERTIES OF LIPIDHEME-MICROSPHERE

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ABSTRACT

Triglyceride microsphere emulsified with phospholipid derivative of heme (5,10,15,20-tetrakis[α,α,α,α-*o*-[2,2-dimethyl-20-[2-(trimethylammonioethoxy) phosphonatoxy]eicosanamido]phenyl]porphinatoiron(II); lipidheme) provides a totally synthetic artificial red cell (lipidheme-microsphere; LH-M). Its structure, solution properties and O_2 binding ability are described. The particle diameter of the LH-M was *ca.* 90 nmφ elucidated by electron microscopy. Viscosity of the LH-M suspension (~1.5 cP) was much lower than that of human blood and the viscosity of mixed system of LH-M/human blood (1/1(v/v)) was 2.5 cP. Specific gravity, osmotic pressure, and colloid osmotic pressure of the LH-M suspension also satisfied the physiological needs. The LH-M can bind O_2 reversibly in response to O_2 pressure ($P_{50}(O_2)$: 41 torr (pH 7.4, 37°C)). O_2 solubility of the LH-M was more than that of human blood caused by its high heme concentration.

INTRODUCTION

Oil-in-water (O/W) lecithin emulsions (lipid microsphere) has already been used in clinics for parenteral nutrition and as a carrier of lipophilic drugs [1-3]. The lipid microspheres are well tolerated by the body since they resemble chylomicrons, and low incidence of side effects have been recognized. We recently have found that O/W lipid microsphere which formulated from triglyceride and

emulsified with amphiphilic heme derivatives; 5,10,15,20-tetrakis[$\alpha,\alpha,\alpha,\alpha$
-o-[2,2-dimethyl-20-[2-(trimethylammonioethoxy)phosphonatoxy]eicosanamido]
phenyl]porphinatoiron(II) (FIG. I, abbreviated as lipidheme) [4-7] as surfactant,
give red-colored dispersion which is able to bind oxygen reversibly in aqueous
medium [8,9]. We report herein structure, solution properties and O_2 binding
ability of the novel totally synthetic red blood cell substitute (LH-M).

MATERIALS AND METHODS

Preparation of Lipidheme-Microsphere

Lipidhemin (Fe(III) met-heme), 1-stearylimidazole (SIm), soybean oil
(SO), and glycerine ([hemin]=0.01~30 mM, 1/0.2/3/ (wt ratio), lipidhemin/SIm =
1/2.5 (mol ratio)) were homogenized in deionized water by ultrasonic generator in
an ice-water bath under nitrogen, to give a red-colored O/W emulsion [9]. After
pH was adjusted to 7.4, the lipidhemin was reduced to iron(II) heme by an addi-
tion of small excess molar of ascorbic acid under nitrogen atmosphere.

Physicochemical Measurement

The particle size of the LH-M was measured by a submicron particle
analyzer. Transmission electron microscopy (TEM) of the LH-M was carried out
by a negative staining method using uranyl acetate. Viscosity of the LH-M sus-
pension was measured with a cone plate rotatory viscometer.

Oxygen Binding Equilibrium

The O_2-binding affinity (O_2 pressure at half O_2 binding for the lipidheme,
$P_{50}(O_2)$) was determined from the spectral changes in visible region at various
partial pressures of oxygen [7].

RESULTS AND DISCUSSION

Preparation and Solution Properties

TEM of typical LH-M ([SO]/[heme]: 3 (wt/wt), 2% glycerine aqueous
dispersion) is shown in FIG. 1. The LH-M looked like spherical particles with
diameters of 80~100 nmϕ, which agreed to the average particle size measured by
dynamic light scattering method using a submicron particle analyzer (85±25
nmϕ).
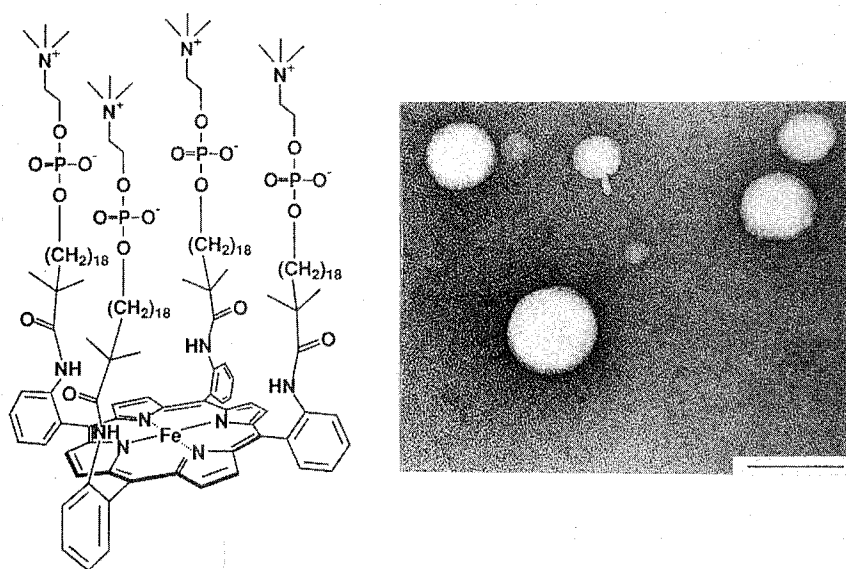
FIGURE 1   Structure of lipidheme and TEM of LH-M (2wt% glycerin solution), bar: 100 nm.

The LH-M suspension was stable and could be stocked for a few months or longer without precipitation and change of the particle size at 4~25 °C. The physicochemical properties of the LH-M are summarized in TABLE I. Specifically, the suspension was characterized by its low viscosity (1.5 cP, [heme]=15 mM), which is much lower than that of human blood (4.5~5.5 cP). This is the most characteristic feature of the LH-M and is a great advantage for in vivo administration.

The specific gravity (d) was almost same as that of saline solution. Osmotic pressure was adjusted to physiological value (280~300 torr) by adding glycerine. Colloid osmotic pressure (COP) of the LH-M suspension itself is very low (~2 torr). It was adjusted to that of human blood (25 torr) by adding of a water soluble polymer, e.g. dextran (Dex, Mw 40,000) or human albumin (Alb). The optimal added amount of these polymers was Dex: 2 wt% and Alb: 5 wt%. Viscosity changes during the addition of these polymers were shown in FIG. 2. The

TABLE I   Solution properties of the LH-M suspension.

	d	Viscosity[a] / cP	OP[b] / mOsm/kg	COP[c] / torr
LH-M[d] [LH]: 5[e]	0.998	1.1	280~300	~2
10[e]	1.001	1.2	280~300	~2
15[e]	1.003	1.5	280~300	~2
Human blood	1.055~ 1.065	4.5~5.0	280~290	25
Saline	1.027	1.3	285	0
Intralipid[R]	0.981	1.5	285	2

[a] At 37 °C. [b] OP: Osmotic pressure. [c] COP: Colloid osmotic pressure.
[d] Dispersed in glycerine solution. [e] mM.
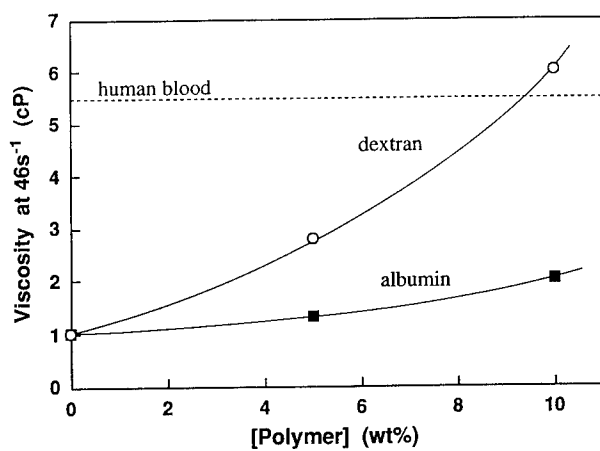
FIGURE 2   Viscosity changes of the LH-M by an addition of polymers.
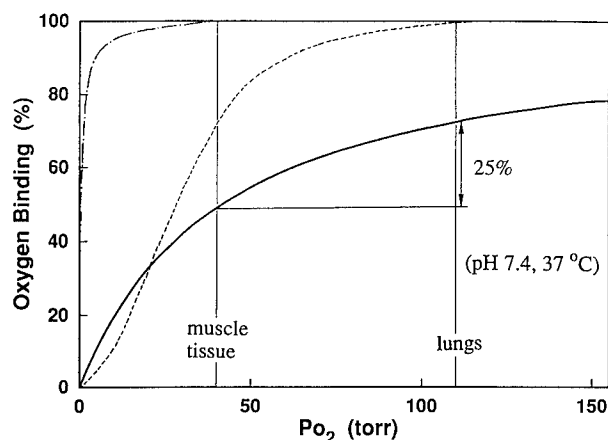
FIGURE 3 O_2 Binding and dissociation curve of the LH-M. Hb in red blood
cell; ------, Mb; —·—.

viscosity did not rise to that of human blood by the addition of Dex; 2 wt% or
Alb; 5 wt%.

Blood compatibility of the LH-M suspension was preliminarily estimated
by viscosity measurement of the blood mixed solution. Even the LH-M and
human blood were mixed with 1/1 (v/v), aggregation and/or precipitate were not
detected and its apparent viscosity was maintained at 2.5cP (at share rate: 230 s^{-1}).
This result suggests that the LH-M has a high blood compatibility.

The other important feature of the LH-M is to soluble the heme at high
concentration in an aqueous medium. That is, the LH-M suspension ([heme]: 15
mM) is able to uptake O_2 gas up to 35 ml/100 ml medium, which is much higher
than that of human blood. Therefore small size of particles (about 80 times small-
er in diameter than that of red blood cells), high oxygen solubility, and low vis-
cosity of the LH-M facilitate the circulation and would permit a better exploita-
tion of collateral microcirculation.

Oxygen Transporting Ability

The visible absorption spectrum of the deoxy heme (λ_{max}: 429, 536 and
562(shoulder) nm) changed to that of oxy heme rapidly on exposure to oxygen
(λ_{max}:424 and 539 nm). The spectrum changed reversibly dependent on O_2 pres-

sures. The $P_{50}(O_2)$ of the LH-M was determined by the spectral changes in response to O_2 partial pressure. While Hill coefficient (n) of this system was 1.0, $P_{50}(O_2)$ was 41 torr at 37 °C (FIG. 3) and appropriate to act as an efficient O_2-carrier under physiological conditions which transports O_2 from the lungs $(P(O_2)=ca.$ 110 torr) to the muscle tissue $(P(O_2)=ca.$ 40 torr) as does Hb. The oxy-heme was slowly degraded to the lipidhemin (Fe(III) met-heme) and this time-degradation curve obeyed first-order kinetics. Life-time (half-life, τ_{50}) of the oxy-heme of the LH-M under physiological conditions was 14 hr.

These results indicated that the LH-M has a potential to act as a novel totally synthetic artificial red cell.

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CHARACTERISTICS OF Hb-VESICLES AND ENCAPSULATION PROCEDURE

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ABSTRACT

The performance of Hb-vesicles depends on the weight ratio of Hb to lipid ([Hb]/[Lipid]). This value is improved by lowering the number of bilayer membrane of the vesicle and raising the concentration of Hb in the interior of the vesicle. Maximum [Hb]/[Lipid] ratio was obtained at *ca.* pH 7, that would relate to the isoelectric point (pI) of Hb at 25 °C. On the other hand, the [Hb]/[Lipid] ratio decreased with ionic strength and increased with lowering temperature. The Hb-vesicles encapsulating 40 g/dl Hb with only one bilayer membrane were isolated by using the difference in the density of the vesicles.

INTRODUCTION

A Hb-vesicle encapsulates concentrated SFHb with thin lipid membrane like a RBC [1-5]. The size is strictly controlled to 0.2 $\mu m\phi$ by extrusion. The performance of Hb-vesicles depends on the [Hb]/[Lipid] ratio, which is determined from the concentration of Hb in the interior of a vesicle ($[Hb]_{in}$) and the number of bilayer membrane (n). Since the surface of the lipid membrane and Hb are kinds of electrolytes, $[Hb]_{in}$ and n should be the functions of pH, ionic strength and temperature. In this report, the solution conditions of Hb which give the high [Hb]/[Lipid] ratio are described. Furthermore, since the Hb-vesicles with

the high [Hb]/[Lipid] ratio have high density, separation with ultracentrifugation by using the difference in the density of the Hb-vesicles is studied.

MATERIALS AND METHODS

Preparation of Hb-vesicles: A mixed lipid powder of 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (DODPC) / cholesterol / stearic acid (SA) (7/7/2 by mol) was prepared by freeze-drying from a benzene solution. A Hb solution was purified from outdated conc. RBC, which was carbonylated in advance, by mixing with organic solvent and heating [6]. After dialysis and adjustment of pH and ionic strength, the Hb solution was ultrafiltrated to *ca*. 45 g/dl. Large multilamellar vesicles prepared by dispersing the lipid powder into a Hb solution were extruded through polycarbonate membrane filters with reducing the pore size to 0.2 μm ϕ. Free Hb was removed by GPC (Sepharose CL-4B).

Evaluation of Hb encapsulation [5]:   The volume of an inner aqueous phase of one vesicle (V_{in}) and the number of molecules in one vesicle (N) are functions of the number of bilayer membrane and the radius of a vesicle (r). The volume of vesicles per unit weight of lipids ($V_{calc.}$ (cm^3/g)) is represented from r, N and the average molecular weight of mixed lipids (M). On the other hand, when the weight of lipid and the total volume of vesicles are m (g) and V(cm^3), respectively, the volume of vesicles per unit weight of lipids ($V_{meas.}$) is V/m. V is obtained by comparing the concentration of glucose in the exterior of vesicles to the apparent glucose concentration. Supposing $V_{meas.} = V_{calc.}$, the number of bilayer membrane (n) is calculated if r is given. The Hb concentration of the interior of a Hb-vesicle ($[Hb]_{in}$) was calculated by introducing the measured [Hb]/[Lipid] ratio.

Separation of Hb-vesicles by using the difference in the density: The density of Hb-vesicles having each n and $[Hb]_{in}$ was estimated by measuring the density of the mixed lipid membrane and Hb solution of each concentration. Hb-vesicles were dispersed in a Hb solution whose density (1.084 g/cm^3) was between the densities of unilamellar and bilamellar Hb-vesicles, and then ultracentrifuged (250,000g, 120 min) to yield fractions of Hb-vesicles with various densities.

RESULTS AND DISCUSSION

Influence of Solution pH [7]:   A maximum of the [Hb]/[Lipid] ratio was observed at *ca.* pH 7, which was almost the same as pI of Hb at 25 °C. The number of bilayer membrane (n) decreases because electrostatic repulsion between negatively-charged surfaces of the membrane becomes strong at high pH. On the other hand, negatively-charged Hb above the pI should receive an electrostatic repulsion from the negatively-charged surface. This causes the lower encapsulation of Hb into vesicles, *i.e.*, lower $[Hb]_{in}$. The repulsion decreases with lowering pH. When the pH becomes lower than pI, electrostatic interaction between positively-charged Hb and the negatively-charged surface occurs in turn. This change would lead to a drastic increase in $[Hb]_{in}$. Therefore, two opposite contributions of n and $[Hb]_{in}$ to the [Hb]/[Lipid] ratio would result in the maximum of the [Hb]/[Lipid] ratio.

Influence of Ionic Strength [7]:   The [Hb]/[Lipid] ratio decreases with NaCl concentration. This can be explained in terms of the larger number of bilayer and the lower $[Hb]_{in}$ with salt concentration. The shielding effect of ions from the added salt on the electrostatic repulsion between negatively-charged surfaces results in an increase in the number of bilayer membrane. From the same reason, electrostatic interaction between bilayer membrane and positively-charged Hb would be decreased by this shielding effect, leading to the lower $[Hb]_{in}$.

Influence of Temperature: The [Hb]/[Lipid] ratio of 1.5 at 4°C decreases almost linearly with elevating temperature (FIG. 1). $[Hb]_{in}$ increases slightly from 25 to 27 g/dl when preparation temperature elevates from 4 °C to 11 °C, which would be due to the decrease in the solution viscosity. On the other hand, the number of bilayer increases with preparation temperature. The pH of the Hb solution (40 g/dl) increased from 7.0 to 7.4, when temperature was reduced from 22 °C to 4 °C. This would be explained from the protonation of dissociated moieties of Hb. The dissociation of fatty acid of bilayer membrane is enhanced by such a pH increase, resulting in the smaller number of bilayer membrane by electrostatic repulsion between negatively-charged surfaces [8].
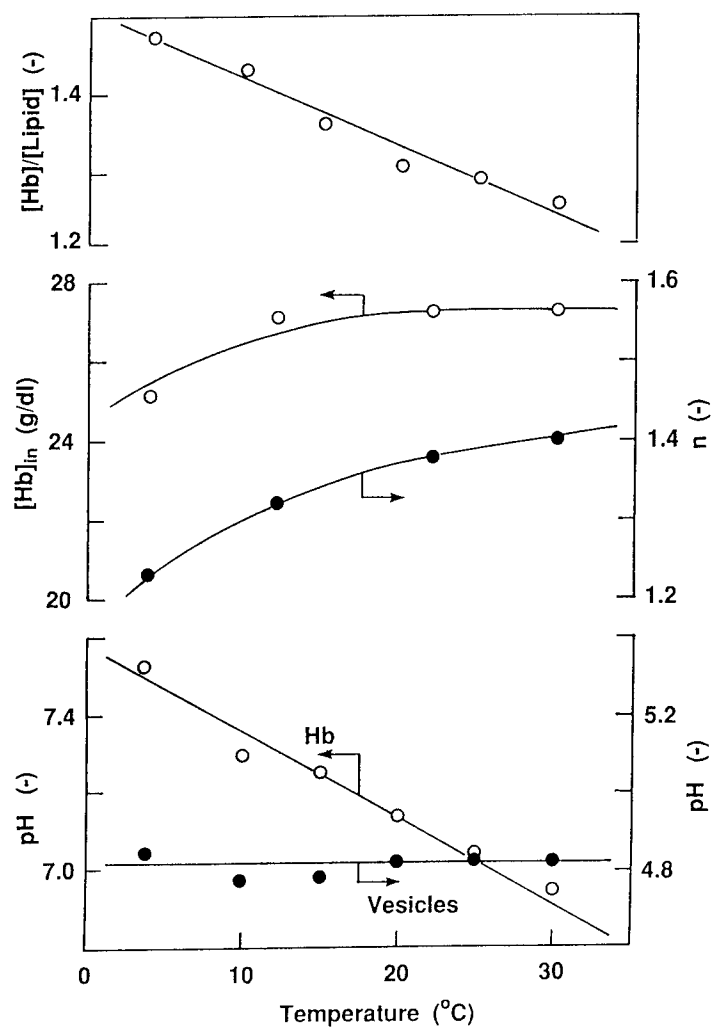
FIGURE 1 Temperature dependence of Hb encapsulation with lipid membranes and the change of pH in a Hb solution and a suspension of a vesicles.
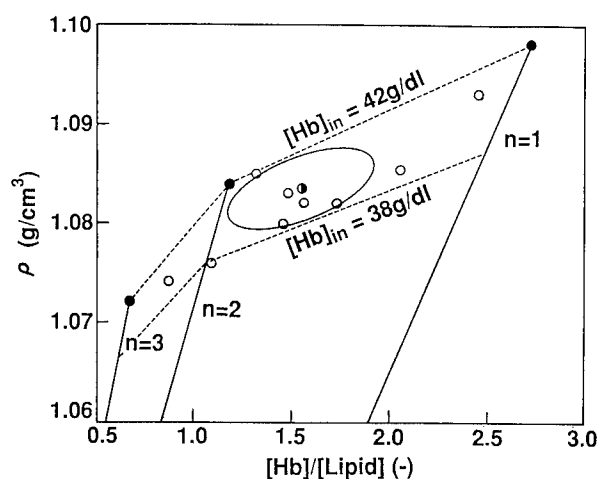
FIGURE 2   The density and the [Hb]/[Lipid] ratio of Hb-vesicles fractionated by ultracentrifugation. The Hb-vesicles with the highest [Hb]/[Lipid] ratio (2.4) was obtained by ultracentrifugation when the density of the fluid was adjusted to 1.084 g/cm^3.

Separation of Hb-Vesicles with a High [Hb]/[Lipid] Ratio: The [Hb]/[Lipid] ratio directly relates to the density of a Hb-vesicle because the density of the concentrated Hb solution is higher than the density of lipid membrane. FIGURE 2 shows the density and the [Hb]/[Lipid] ratio of Hb-vesicles fractionated by ultra-centrifugation. Solid lines are the calculated relationship with constant [Hb]$_{in}$ or n. The loop indicates the distribution for Hb-vesicles before ultracentrifugation. The Hb-vesicles of which [Hb]/[Lipid] ratio was 2.4 were isolated as a pellet when the density of the fluid at centrifugation was adjusted to 1.084 g/cm^3. The value means that the Hb-vesicles of which average size is 0.2 μmφ encapsulate about 40 g/dl of concentrated Hb with one bilayer membrane.

CONCLUSION

The preparation of Hb-vesicles was influenced by solution conditions of Hb at extrusion. The Hb-vesicles with the high [Hb]/[Lipid] ratio were prepared at pH 7 (25 °C), low ionic strength and low temperature. Furthermore, the Hb-vesi-cles with the higher [Hb]/[Lipid] ratio were separated by using the difference in

the density of the Hb-vesicles. Thus obtained Hb-vesicles were 0.2 μmϕ size and encapsulated 40 g/dl Hb with only one bilayer membrane.

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PREPARATION AND CHARACTERISATION OF POLY(LACTIC ACID) HEMOGLOBIN MICROSPHERES

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ABSTRACT

For many years, a lot of research effort has been carried out with a view to preparing blood substitutes. Our group has developped a process of encapsulation of hemoglobin in polylactid microspheres.

An aqueous solution of hemoglobin was emulsified into a solution of polymer in methylene chloride to form a W/O emulsion. This primary emulsion was then added to a external aqueous phase under stirring until the evaporation of methylene chloride. The microspheres were separated by filtration and washed with distilled water. Microspheres were spherical and their sizes vary between 10 and 500 µm. More than 80 % of the hemoglobin was encapsulated. From the absorption spectra of hemoglobin from microspheres, we did not notice any alteration of the oxygen carrier. The dissociation curve of the hemoglobin demonstrated the permeability of the polymeric wall of these microspheres to oxygen. This curve was relatively sigmoidal and presented a P50 similar to that of free hemoglobin in the same experimental conditions. A cellulose's acetate gel electrophoresis of hemoglobin extracted from the microspheres showed one band that correlates with intact hemoglobin. These results suggest that hemoglobin does not interact chemically with the polymer matrix and that the process of microencapsulation does not alter the hemoglobin molecule.

INTRODUCTION

The hemoglobin solution is generally presented as a blood substitute or "artificial blood". However, free hemoglobin possesses serious limitations represented mainly by its relatively short vascular retention time [1,2] and its high affinity for oxygen [3]. To overcome these limitations, our purpose has been to develop synthetic membrane in order to shape then into a sort of artificial cells. The microencapsulation of hemoglobin has already been studied by many authors: Chang was the first to prepare hemoglobin microcapsules through interfacial polymerization (nylon 6-10) or coacervation processes (collodion) [4].

In 1980, Ndong-Nkoume et al in laboratory obtained similar results through interfacial polymerization between sebacoyl chloride and hexamethylene diamine [5].

In line with the previous studies on microencapsulation, the membrane of our microsphere is constitut made up of poly(lactic acid) which is known as biocompatible and biodegradable. In the present study, hemoglobin has been incorporated into microspheres using a multiple emulsion technique. Our objective was to prepare microspheres, to determine their physico-chemical properties and the functional state of hemoglobin before and after microencapsulation.

MATERIALS AND METHODS

Materials

Human hemoglobin was purchased from the Nancy Blood Tranfusion Center. Poly(L-lactic acid) with an inherent viscosity of 1.28 g/dl in chloroform was obtained from Medisorb. Polyvinyl alcohol (Mw 10000 to 30000) was purchased from Sigma. Dichloromethane was obtained from Fluka.

Microsphere preparation

It was based on the double emulsion method [6] : An aqueous solution of hemoglobin (1.7 ml at 300 g/l) was emulsified into a solution of poly(L-lactic

acid) (0,5 g) in dichloromethane (20 g). The primary emulsion was then added to an aqueous phase containing 0.25 per cent polyvinyl alcohol. The resulting emulsion was agitated by mechanical stirring at 1500 rpm during 1 hour at room temperature until the evaporation of the solvent. The microspheres were finally separated by filtration, washed with water and then stored at +4°C.

Microspheres characterization

The structure and surface morphology were studied by light microscopy (Axioskop Zeiss) and scanning electron microscopy (Cambridge Instruments) (Faculty of Medecine) at different magnifications. The particle size was measured using a Master Sizer instrument (Malvern).

The amount of hemoglobin into the microspheres was determined indirectly by measuring the unentrapped residual hemoglobin in the supernatant by spectrophotometry (UV 160 Shimadzu Spectrophotométer), the absorbance beeing measured at 540 nm by Drabkin's method. To find out whether the encapsulation process was likely to denature hemoglobin, we studied : the absorption spectra of hemoglobin microspheres from 500 to 700 nm (UV 160 spectrophotometer) and the oxygen dissociation curve with a Hemox Analyzer System (T.C.S) in 50 mM, pH 7.4 Bistris buffer at 37 °C. The values of P50 were taken directly from the graphs and the Hill's coefficients n were calculated as the first derivatives of the curves. The electrophoresis of encapsulated hemoglobin after its extraction and of unencapsulated control hemoglobin were studied on cellulose acetate (Cellogel and Celloprofil Sebia).

RESULTS AND DISCUSSION

Poly(lactic acid) microspheres containing hemoglobin appear spherical in shape, well individualised and not aggregated (fig.1). Their size distribution ranging from 10 to 500 μm is shown in figure 2. This distribution shows that the diameter of more than 50 % of the microspheres is lower than 150 μm. The amount of hemoglobin encapsuled was more than 80 % of the hemoglobin used.

From the absorption spectra of hemoglobin from microspheres, we did not notice any alteration of the oxygen carrier : it presented the two classical bands at 577
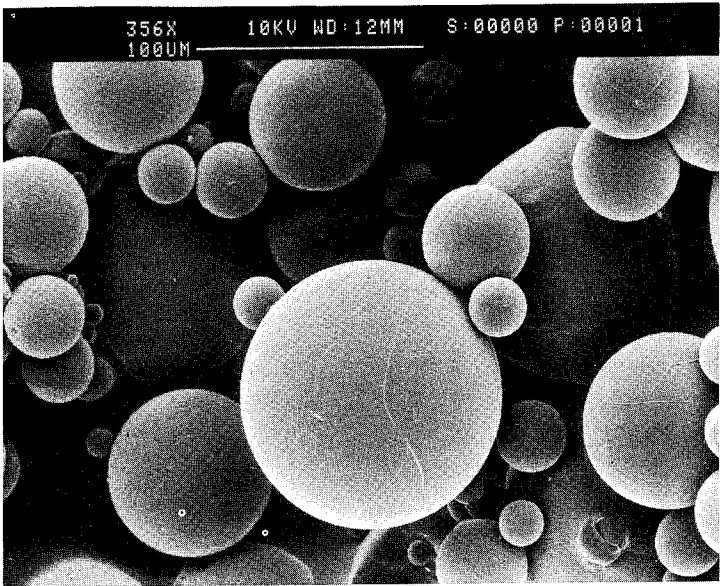
FIGURE 1: Scanning electron micrograph of hemoglobin microspheres.
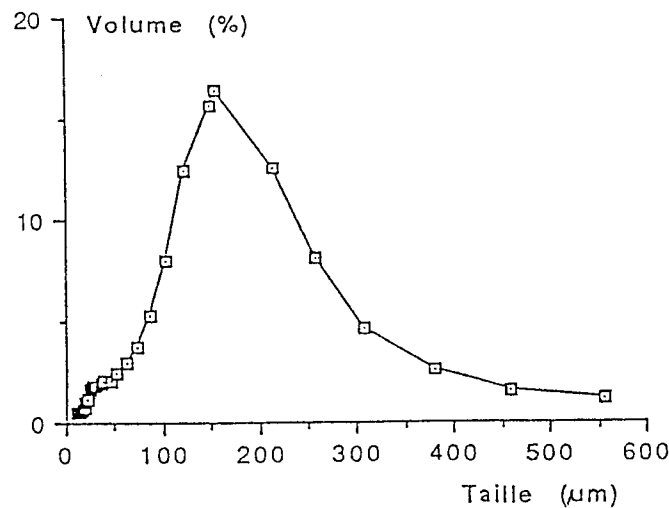
FIGURE 2: Exemple of size distribution of microspheres.
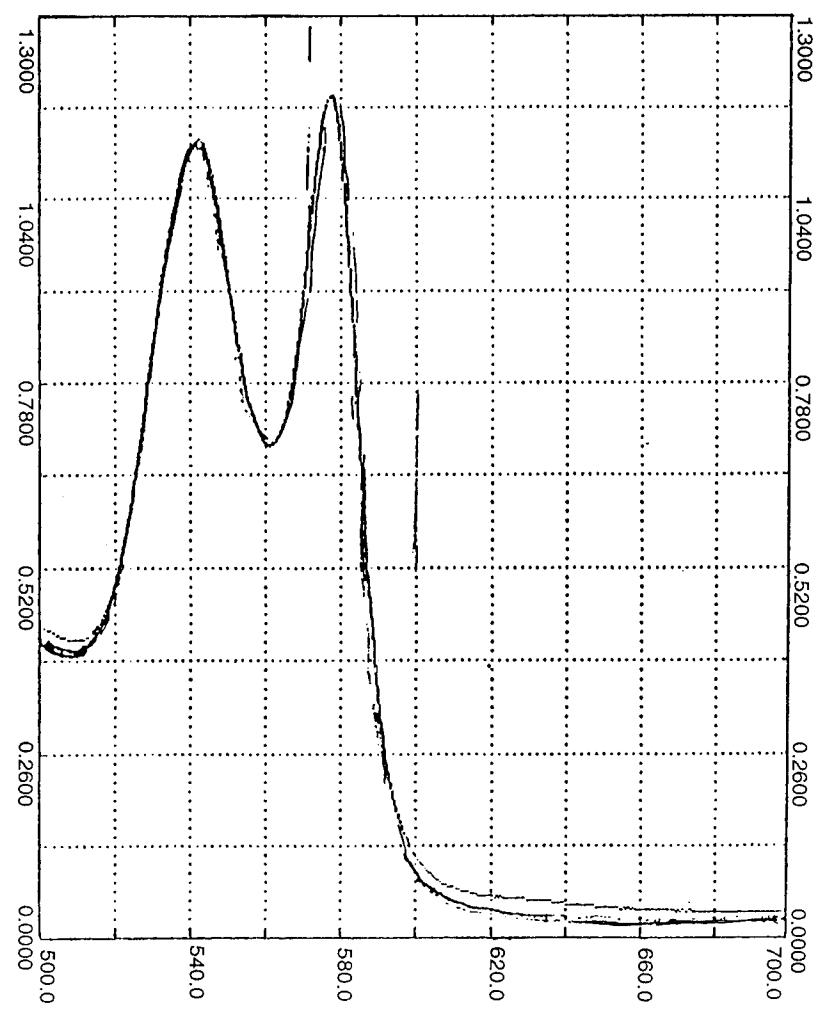
FIGURE 3: Visible absorption spectra of hemoglobin from microspheres and
of control hemoglobin
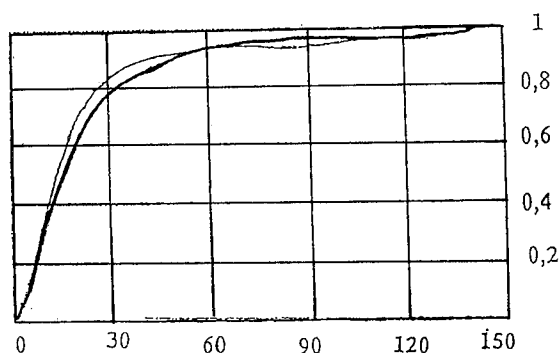
FIGURE 4: Oxygen dissociationcurves of control (p50=13.8 mmHg) and
hemoglobin from microspheres (p50=13.9 mmHg).

and 540 nm. Figure 3 shows the lack of difference between the control
hemoglobin and that from the microspheres.

The oxygen dissociation curve of the microspheres containing hemoglobin
demonstrated that hemoglobin still possesses its capacity to bind reversibly with
oxygen despite the process of microencapsulation (fig. 4).

Encapsulated or extracted hemoglobin presented relatively sigmoidal curves with
values of P50 approximately 13.9 mmHg (free hemoglobin approximately 13.8
mmHg). Hill's plot values of hemoglobin was 2 compared to 2.5 for control
hemoglobin in the same experimental conditions. The cellulose acetate gel
electrophoresis of the extracted hemoglobin was identical to that of control
hemoglobin. All these results suggest that the process of microencapsulation does
not alter the hemoglobin molecule.

To our knowledge, this technique of hemoglobin encapsulation had never been
used before and yielded here very encouraging results.

Our microspheres are spherical, well individualised and this method allows a rate
of incorporation superior to 80 % of the hemoglobin used. In the literature, this
rate does not generally exceed 30 %. The functional properties of encapsulated
hemoglobin remain virtually unchanged and the microspheres appear to be well
preserved .

In conclusion, the preparation of hemoglobin microspheres was conceptually simple and reproducible. Our objective is to reduce their size to create a kind of artificial cells.

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CHARACTERISTICS OF *NEO RED CELLS*, THEIR FUNCTION AND SAFETY : *IN VIVO* STUDIES.

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ABSTRACT

A new type of artificial oxygen carriers, the Neo Red Cells (NRCs) have been developed and investigated for oxygen transporting efficiency and safety in experimental animals. Stroma free hemoglobin from outdated human red blood cells together with inositol hexaphosphate as an allosteric effector under sterile, pyrogen free condition were encapsulated in liposomes and then were coated with polyethylene glycol bond to hydrogenated soy phosphatidylethanolamine as a surface modifier to prevent aggregation of NRCs in plasma. The efficiency of the NRCs in tissue oxygenation was studied in rabbits which were made severely anemic by drawing 85% of their blood and immediately replacing it with NRC solution. The animals, all recovered to pre-anemic conditions within 6-8 hr and lived normally until being sacrificed, 6 months after the exchange transfusion. The circulation half-life and tissue distribution of NRCs were studied using radiolabeled NRCs. Within the circulation, the half-life of NRCs was 21 hr and extravascularly, they were distributed mainly in and metabolized by the reticuloendothelial system within 7 days.

Our observations suggest that the NRCs prepared and investigated in this study are efficient oxygen carriers without causing serious adverse reactions and can be prepared free from pathogenic micro-organisms by special filtration technique before encapsulation of Hb. Currently, experiments are ongoing to control auto-oxidation of oxyHb to metHb which is higher in NRCs than in native red cells at physiological conditions.

INTRODUCTION

Over the past 50 years, various types of artificial oxygen carriers have been developed to replace transfusion of native human red cells and avoid the risk of transfusion-related infection. In recent years, several types of hemoglobin(Hb) encapsulating liposomes have been studied to function as artificial red blood cells. Typical examples are "Hemosomes" by Djordjevich and Miller[1], "Liposome-encapsulated hemoglobin (LEH)" by Farmer and Gaber[2] and "Neohemocytes (NHC)" by Hunt et al.[3].

In this paper, we report on the newly developed hemoglobin encapsulating liposomes called "Neo Red Cells (NRCs)" and describe their oxygen transporting efficiency and safety in experimental animals.

MATERIALS AND METHODS

Preparation of NRCs

Stroma free hemoglobin (SFH) solution was prepared from outdated human red blood cells (Japan Red Cross) by standard procedures, then filtered through 300K-Da cut off filters (pore size 5nm) to remove the stroma. Using ultrafiltration, the SFH solution was concentrated up to 45gHb/dl. A lipid mixture(360g, Presome, Nippon Seika) of hydrogenated soy phosphatidylcholine, cholesterol, myristic acid and α-tocopherol in the ratio of 7:7:2:0.28 respectively was hydrated with a small amount of water at 60°C. The homogenous hydrated lipids were then added to concentrated SFH (2L) and inositol hexaphosphate (IHP) as an allosteric effector in a molar ratio of IHP:Hb=0.8:1. The mixture was placed into a microfluidizer (Micro Fluidics Corp.) to form NRCs. Extravesicular Hb was removed by twice centrifugation at 5000xg for 15min in the presence of 6% hydroxy ethyl starch (HES) as a liposome aggregating reagent. The resulting NRC suspension was harvested by filtration through 0.45μm and then through 0.2μm filters and finally concentrated to 7gHb/dl by ultrafiltraion. NRCs thus prepared have a tendency to aggregate in plasma. To prevent aggregation, 0.1% polyethylene glycol (Mw=5000) was covalently bound to hydrogenated soy phosphatidylethanolamine (PEG-PE: synthesized in our laboratory) as a liposomal surface modifier.

Measurement of Circulation half-life of NRCs

[14]C-dipalmityl phosphatidylcholine (^{14}C-DPPC, 5.55Mbq, Amersham) was mixed with Presome (2.7g). The ^{14}C-labeled NRCs were then prepared with the lipid mixture. Radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb 1900CA). The specific radioactivity of ^{14}C-labeled NRCs was 3.024μCi/ml(5gHb/dl). Male Wister rats (n=3.6 weeks, 150-200g) were infused with 1, 3, 10, 20 and 30ml/kg of ^{14}C-labeled NRCs and their clearance was determined by drawing a 100μl of blood from tail vein.

Determination of biodegradation of NRCs

Male ICR mouse (n=3.5weeks, 27-29g) were infused with 20ml/kg of NRCs, and then sacrificed at 1, 3, 5, 7 days postinfusion. Liver and spleen were removed, homogenized

by POLYTRON and their NRC-derived Hb content was measured using an adaptation of the method of enzyme-immuno-assay (Labozyme FECA-EIA, Labosystems).

Exchange transfusion (ET)

Male Japanese white rabbits (n=5, 2.8-3.0kg) were anesthetized with pentobarbital . Bleeding was carried out twice from the left femoral artery at the rate of 8-10ml/min. The first bleeding was 25ml/kg and the second was 20ml/kg. This was immediately followed by infusion of an equal volume of 6% HES solution. Finally, a further 20ml/kg blood was removed. With this procedure the rabbits were made severely anemic(hematocrit <5%). They were then infused with 40ml/kg of NRCs; this reflected 85% ET. Apart from infusion of NRCs, the anemic rabbits were not assisted by any other means. Blood gas chemistry, A-V blood pressure, heart rate, cardiac output, lactate and blood glucose levels were measured during the experiments. The oxygen transporting rate (ml/min, OTR) was calculated from the following formula:

$$OTR \ (ml/min) = A\text{-}V \ O_2 \ content \ (ml/dl) \ x \ Cardiac \ output \ (dl/min)$$

RESULTS

Physicochemical properties and O_2 carrying capacity of NRCs

The physicochemical properties and O_2 carrying capacity of NRCs are summarized below.

TABLE 1 : Physicochemical properties and O_2 carrying capacity of NRCs.

Property	NRCs	Human RBCs
Hemoglobin (g/dl)	15	14-16
Total lipid (g/dl)	8.5	0.5
Vesicle diameter (μm)	0.18-0.22	5-8
Initial MetHb (%)	5>	1>
Increase in MetHb (%/month)[a]	1.5	1>
P_{50}(mmHg)	45	26
Hill coefficient	1.9	2.8
AV O_2 content[b](ml/dl)	7.3	5.6
Half-life (hr)	21[c]	12-1440[d]

a: stored at 4°C, b: The volume of O_2 that could be delivered to tissues by the suspensions was calculated assuming arterial and venous PO_2 values to be 100 and 40mmHg respectively, c: The administered dose of NRCs was 30ml/kg in rats, d: Depending on storage time.

The circulation half-life of NRCs.

The clearance profile of NRCs is shown in figure 1. It can be seen that NRCs are cleared from the circulation in two phases, a rapid (3 hr) phase followed by a slow phase, lasting over 50 hr. The circulation half-life of NRCs for doses of 1, 3, 10, 20, 30 and 30(on ET) ml/kg were 1.1, 1.6, 4.1, 6, 21 and 30 hr respectively, showing that the half-life of NRCs increases with the administered dose.

The residual NRC-derived Hb in organs.

The residual NRC-derived Hb in the liver and spleen are shown in figure 2. The Hb in the liver and spleen was degraded within 7days after the administration of NRCs.

Observations after exchange transfusion (ET)

In severely anemic rabbits prepared for ET, the hemoglobin concentration of the animals fell from 11.8g/dl to 1.6g/dl and the hematocrit from 37% to 5% . If the ET was carried out with 6%HES-saline solution instead of NRCs, rabbits were dead within 6hr due to anemia, but when they were infused with NRCs, they lived normally up to 6 months at which time, they were sacrificed. Further, the oxygen partial pressure of arterial and venous blood remained within normal levels after infusion of NRCs. The blood glucose and lactate levels were transiently increased by ET, and then returned to normal levels within 7-8hr. The cardiac output was also increased transiently from 0.5L/min to 0.7L/min following ET. The metHb concentration increased by 20% at 24 hr after ET due to inefficiency of metHb reductase in NRCs. The $P_{50}O_2$ value decreased slightly, from 45mmHg to 40mmHg. The OTR of NRCs together with that of own (non-infused) blood is shown in Figure 3. As shown, the OTR of NRCs was reduced to 50% at 48hr post-infusion (B), but after 24 hr, OTR of own blood began to increase such that the animals could become independent of NRCs, with full recovery within 2weeks. The NRCs were extensively phagocytosed by Kupffer's cells in the liver and splenocytes but we found no histological sign of damage in these cells.

DISCUSSION

In this report, we have described preparing a new type of liposome-encapsulated hemoglobin (NRCs) to function as oxygen carriers. One major problem was their tendency to aggregate (form clumps) in the plasma which we successfully overcome by coating them with a surface modifier (PEG-PE). Application of rigorous filtration techniques to the preparation of stroma free Hb can ensure that the NRCs are free from infectious micro-organisms.. To investigate their efficiency as oxygen carriers for tissue oxygenation, we prepared animal model of anemia which was as severe as hemorrhagic shock such that the animals could not survive without transfusion of oxygen carriers. The anemic animals, when infused with NRCs, recovered to pre-anemic conditions, supported by NRCs until the recovery of their own red cells and lived until they were sacrificed. We observed that the rate of oxygenation of NRCs in the lungs and deoxygenation in the tissues were much faster than that of normal human red cells (data not shown), primarily
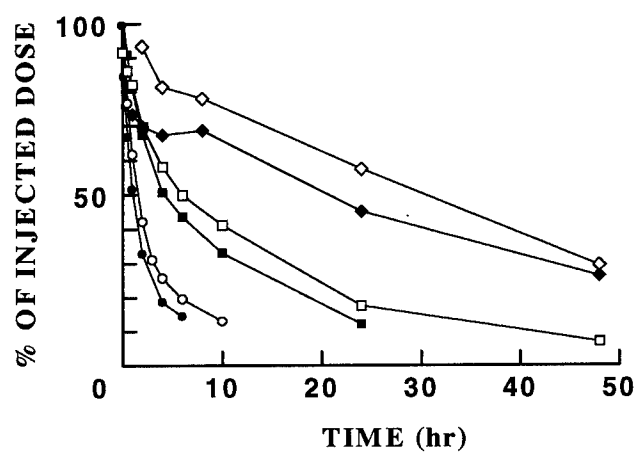
FIGURE 1. The clearance from the circulation of ^{14}C-labeled NRCs, at different doses.

● : 1ml/kg   ○ : 3ml/kg   ■ : 10ml/kg   □ : 20ml/kg

◆ : 30ml/kg   ◇ : 30ml/kg (on exchange transfusion)
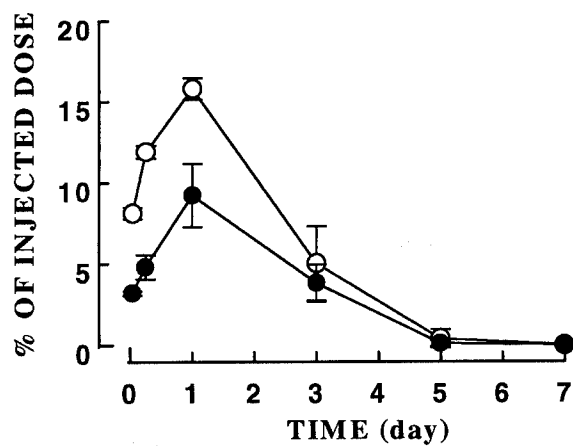
FIGURE 2. Biodegradation of NRCs in spleen and liver as determined by enzyme-immuno-assay of NRC-derived Hb.

● : Spleen   ○ : Liver
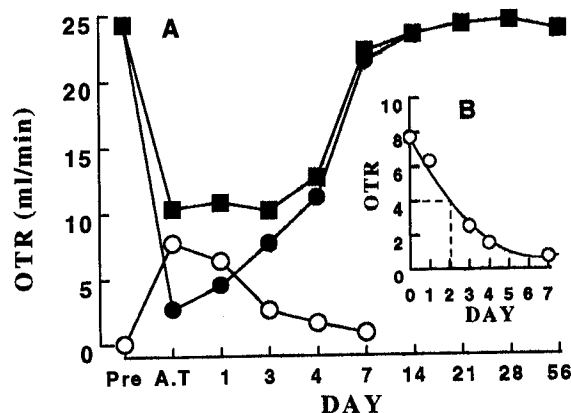
FIGURE 3. The oxygen transporting rate (OTR) of NRCs and own (rabbit) red blood cells after exchange transfusion (A) and the decline in OTR of NRCs with time after exchange transfusion (B). (A.T : After transfusion)

○ : NRCs   ● : Rabbit RBCs

■ : Combination of rabbit RBCs and NRCs.

because of their smaller size but much greater surface area to volume ratio as compared with native human red cells.

The ultimate aim is that NRCs to be used as an alternative to native human red cells in emergency blood transfusion because, in addition to being a much more efficient oxygen carriers than native red cells, they have also a longer storage life (about 12 months). However, in vivo, NRCs are rapidly degraded, mainly in the liver and spleen but at a rate which allows it to be replaced by body's own red cells. This can be seen in figure 3 that as the OTR of NRCs declines with time, OTR of animals' own red cells starts to rise, reflected in the rise of the combination OTR.

In conclusions, NRCs are efficient oxygen carriers which can be prepared free from pathogenic micro-organisms. They are cleared from the circulation at a rate which, in some conditions may be compensatable by body's own hemopoietic process. At present, experiments are underway to control auto-oxidation of oxyHb to metHb which is higher in the NRCs than in native red cells at physiological conditions.

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TOXICITY OF LIPOSOMES CONTAINING LOW MOL% OF DIENOYL PHOSPHOCHOLINE TO BLOOD: USE OF CARBOXYMETHYL CHITIN TO REDUCE TOXICITY.

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ABSTRACT

Toxic effects of liposomes composed of the synthetic lipid, 1,2-bis(octadeca-2,4-dienoyl)-sn-glycero-3-phospho-choline (C_{18}DENPC) and cholesterol (Cho) were studied. In the present work, we have explored, 1) fusion between C_{18}DENPC/Cho-liposomes and erythrocyte ghost (EG) membranes with resonance energy transfer assay, 2) hemolysis induced by C_{18}DENPC/Cho-liposomes, and 3) turbidity changes in native plasma on contact with C_{18}DENPC/Cho-liposomes, in the presence or absence of carboxymethyl chitin (CM-chitin). In the absence of CM-chitin, extents of fusion, hemolysis and turbidity changes in native plasma increased with the decrease in C_{18}DENPC content. In the presnce of CM-chitin at a concentration of 10^{-3} or 10^{-2}% (w/v), fusion of C_{18}DENPC/Cho-liposome with EG was inhibited. Extents of hemolysis and turbidity changes in native plasma induced by C_{18}DENPC/Cho-liposomes were reduced depending upon CM-chitin concentration.

INTRODUCTION

Native cell membranes are stabilized by proteins and/or heteropolymeric cell wall components, whereas membrane mimetic chemistry utilizes externally applied crossliking agents or lipid polymerization to stabilize model membrane lipid bilayer structure [1] .

Chitin, a poly-(1-4)-β-2-acetamido-2-deoxy-D-glucopyranose, and its derivatives are expected to be usable as biomaterials because of their nontoxic and enzymatically biodegradable properties. Carboxymethyl chitin (CM-chitin) has been used to stabilize the liposomes composed of phosphatidylcholine [2-6]. Used in this manner, CM-chitin is bound to the surface of the liposomes, thereby strengthening the liposomes and providing increased stability to the liposomes.

In this study, we have explored the effect of CM-chitin on the fusion of liposomes composed of dienoyl phospholipid, 1,2-bis(octadeca-2,4-dienoyl)-sn-glycero-3-phosphocholine (C_{18}DENPC) and cholesterol (C_{18}DENPC/Cho-liposomes) with erythrocyte ghost membranes, and the interactions of C_{18}DENPC/Cho-liposomes with red blood cells and native plasma.

MATERIALS AND METHODS

C_{18}DENPC was synthesized according to the method of Ringsdorf and Dorn. Product was characterized by IR and NMR. Liposomes were prepared by the method described previously [5]. Liposomes were photopolymerized in quartz cuvettes by irradiation with light from a high pressure mercury lamp 240-270 nm. Photopolymerization kinetics were followed by recording diene lipid absorption (257 nm) on a Perkin Elmer Lambda B4 UV/VIS spectrophotometer.

Membrane fusion was followed by monitoring lipid mixing in 5 mM sodium acetate/5 mM Hepes, pH 7.4 (Hepes buffer) with the resonance energy transfer (RET) assay [7]. Liposomes labeled with 0.8 mol% each of N-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)phos-

phatidylethanolamine (N-NBD-PE) and N-(lissamine Rhodamine B
sulfonyl)-phosphatidylethanolamine (N-Rh-PE) were prepared in
Hepes buffer, and mixed with erythrocyte ghosts (EG) or
unlabeled liposomes. After mixing, the increase of N-NBD-PE
fluorescence was followed with a Perkin Elmer Luminescence
Spectrometer LS 50. Excitation wavelength was 465 nm (10 nm
slit), and the emission was recorded at 530 nm (10 nm slit).
The initial fluorescence of the solution was taken as the zero
level. The level of infinite dilution (100 % fluorescence) was
obtained after disruption of the liposomes in Triton X-100 (1 %
w/v) solution.

        Interaction of liposomes with red blood cells (RBC) was
observed in 5 mM sodium acetate/5 mM Hepes/150 mM NaCl, pH 7.4
(Hepes-NaCl buffer). Thus, after 1 hr incubation of the mixture
of liposomes and RBC at 37 °C, intact RBC were recovered in the
pellet by centrifugation (2,300 x g for 10 min), and the amount
of hemoglobin released into the supernatant was analysed
spectrophotometrically [6].

        Interaction of liposomes with native plasma was observed by
monitoring the turbidity of the mixture of liposomes and bovine
plasma at 600 nm where liposomes and plasma individually have
minimum absorbance [6].

        Experiments on membrane fusion, liposomes-RBC interaction
and liposomes-plasma interaction were carried out in the
presence and absence of carboxymethyl chitin (CM-chitin).
Concentration of CM-chitin was determined by the   method
described previously [5]. Liposome-treated EG in the presence
or absence of CM-chitin was observed with a phase-contrast
photomicroscope,   Nikon labophot-2 equiped with Episcopic-
Fluorescence attachment EF-D.

        Differential scanning calorimetry was carried out on a
Perkin Elmer DSC-7 calorimeter. Lipid dissolved in chloroform,
were evaporated under N_2, dried for 1 hr under high vacuum and
lipid dispersions in Hepes buffer were vortexed at 50 °C.
Aliquot samples were sealed in alminiun sample pans. DSC
scans were recorded with a heating rate of 2°C/min.

fusion (%)
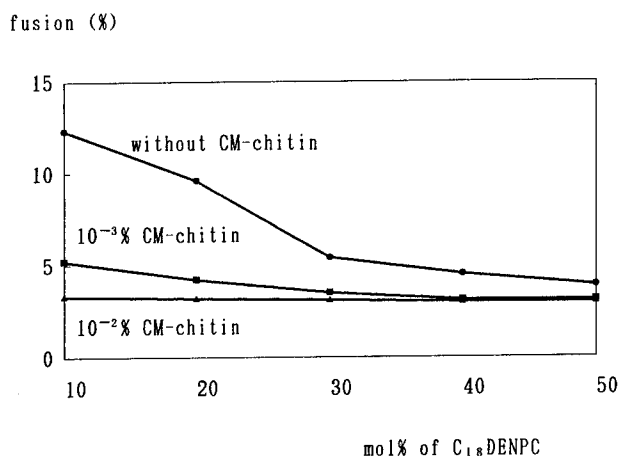
mol% of C_{18}DENPC

FIGURE 1.   Extents of fusion of C_{18}DENPC/Cho-liposomes with
            EG as a function of C_{18}DENPC content at 25°C, pH 7.4.

RESULTS

Phase transition of C_{18}DENPC

C_{18}DENPC phase transition   occurred at 19°C.   The transi-
tion enthalpy of C_{18}DENPC was 39.7 kJ/mol.   Photopolymerization
induced transition broadening $(\geq 10^{\circ}$C) reflecting molecular
heterogeneity of   the   polymer.   Photopolymerized C_{18}DENPC
conserved a pronounced phase transition at approximately 20°C
with a transition enthalpy of 27.2 kJ/mol.

Liposome fusion

Figure 1 shows extents of fusion between C_{18}DENPC/Cho-
liposomes and EG at 25°C and pH 7.4, in the presence and ab-
sence of CM-chitin. In the absence of CM-chitin,   extents of
fusion incresed with the decrease in C_{18}DENPC content.   The
most striking finding is the effects of CM-chitin on the fusion.
In the presence of CM-chitin at a concentration of 10^{-3} or 10^{-2}
% (w/v), fusion was inhibited.
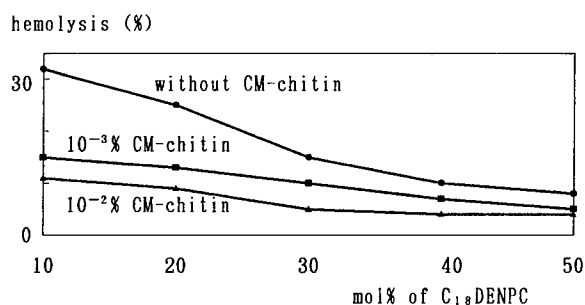
hemolysis (%)

FIGURE 2.   Extents of hemolysis induced by C_{18}DENPC/Cho-liposomes
            as a function of C_{18}DENPC content at 37°C, pH 7.4.
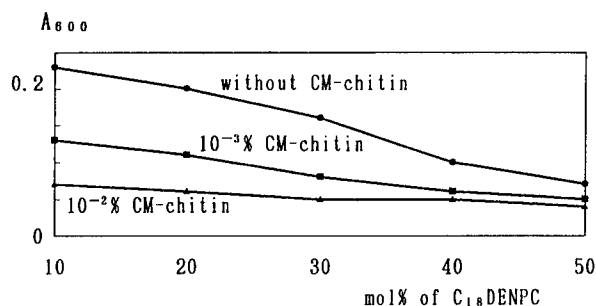
FIGURE 3. Turbidity change in plasma on contact with C_{18}DENPC/
           Cho-liposomes after 1 hr incubation at 37°C, pH 7.4.

Interaction of C_{18}DENPC/Cho-liposomes with RBC and Plasma

    C_{18}DENPC/Cho-liposomes interacted with RBC and caused some
hemolysis in the absence of CM-chitin.   As   shown in   Figure 2,
extents of hemolysis increased with the decrease   in C_{18}DENPC
content.   In the presence of CM-chitin,   extents of   hemolysis
were remarkably reduced.

    As shown in Figure 3, plasma became turbid on contact with
C_{18}DENPC/Cho-liposomes depending upon C_{18}DENPC content.   In the
presence of CM-chitin, the turbidity changes in plasma were re-
duced to 75-50% of those in the absence of CM-chitin.

NISHIYA AND CHANG

DISCUSSION

These experimental results suggest that the liposomes with lower C_{18}DENPC content have higher toxicity to blood components. By an independent experiment, it was shown that the extents of polymerization of the liposomes decreased with the decrease in C_{18}DENPC content, indicating that the C_{18}DENPC monomer is toxic to blood components.

In the EG-C_{18}DENPC/Cho-liposomes fusion experiments, EG-liposome aggregate was observed in the absence of CM-chitin under the microscope, however, in the presence of CM-chitin, small aggregate of liposomes, instead of EG-liposome aggregate, was observed. These observations indicate that, in the absence of CM-chitin, liposomes are solely involved in asymmetric fusion with EG membranes, while, in the presence of CM-chitin, liposomes associate to CM-chitin to form an aggregate and lose the propensity to fuse with EG membranes.

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SUBMICRON BIODEGRADABLE POLYMER MEMBRANE HEMOGLOBIN NANOCAPSULES AS POTENTIAL BLOOD SUBSTITUTES: A PRELIMINARY REPORT

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ABSTRACT

We used biodegradable polymers like polylactic acid, poly-isobutylcyanoacrylate and others to prepare nanocapsules containing hemoglobin. We prepared the optimal mean diameters of between 0.08 to 0.12 micron (80 - 120 nm). They are spherical and homogeneous. The membrane thickness is 0.005-0.015 micron (5-15nm). With different formulation, the hemoglobin contents in the particles may be varied from 30 - 45 %. Phospholipid is not required in bovine hemoglobin. It is required for human hemoglobin in order to retain cofactors required for optimal P50. The P50 of the biodegradable polymer membrane containing bovine hemoglobin was between 27 - 29 mmHg. This is the same P50 as bovine hemoglobin used in the preparation. Thus the procedure of preparation did not damage hemoglobin.

INTRODUCTION

Early attempts to use hemoglobin solution as a blood substitute were limited by such problems as renal damage and rapid removal from ciriculation (1). to overcome these problems, the first artificial red blood cells were prepared by microencapsulating hemoglobin (2-4). Membranes for these early artificial red blood cells included collodion, nylon, other polyamides, crosslinked hemoglobin, phospholipid-cholesterol complexed on caross-linked protein membrane and others (2-4). These artificial red cells with diameters down to one micron survived only for a very short time in the circulation after intravenous injections. The use of submicron phospholipid microcapsules (liposomes) has increased the survival time in the circulation (5-9). The stability and strength of these bilayer lipid vesicles needs to be improved.
Our earlier polylactide biodegradable microcapsules were of micron size (10). The present study is to use a new method to prepare biodegradable polymer membrane hemoglobin nanocapsules with mean diameter in the nanometer range of about 0.08 to 0.180 microns (80-180nm)(11)

BIODEGRADABLE POLYMER MEMBRANE
HEMOGLOBIN NANOCAPSULES

MEAN DIAMETERS:
0.08 TO 0.2 MICRONS
(80 TO 200 NANOMETERS)
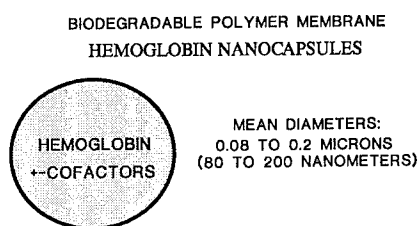
Fig. 1: Schematic representation of biodegradable
polymer membrane hemoglobin nanocapsules.

MATERIALS AND METHODS

Materials:

Polylactic acid (PLA) were obtained from Polysciences Inc. (Canada). Surfactant (Tween 20) and L-α-Phosphatidylcholine (hydrogenated) were from Sigma Chemical Co. (U.S.A.). Dialysis membrane (Spectrapor 5) was purchased from Fisher Scientific Co.. All the other chemicals were of reagent grade.

Preparation of Hemoglobin Solution

Briefly, hemoglobin was obtained by hypotonic hemolysis of bovine red cells and it was made stroma-free by toluene extraction and was clarified by high speed centrifugation. The resulting solution contained 10 - 15 g hemoglobin/dl. In order to minimize the formation of methemoglobin, the manipulation was carried out at 4 °C and the hemoglobin solution was controlled at pH 7.4.

Preparation of biodegradable polymer nanocapsules containing hemoglobin.

100 mg of Polylactic acid and 100 mg of Phosphatidylcholine were dissolved in a mixed solution of ethanol (10 ml) and acetone (15 ml). This solution was slowly injected into 50 ml of 5 g/dl hemoglobin solution containing 0.1 % Tween 20 under constant magnetic stirring. Diffusion of ethanol and acetone into the aqueous phase resulted in the polymer membrane formation. The ethanol and acetone in the aqueous phase can be easily eliminated by dialysis against physiologic saline solution at 4 °C. The hemoglobin nanocapsules was separated by centrifugation and resuspended in saline solution.
Other biodegradable polymers like poly-isobutylcyanoacrylate can also be used. Other lipids such as DSPC, DPPC and other phospholipids may be used. With different polymer and different lipids, the average particle size of the preparation can be varied from 80 to 390 nm.

Steady shear viscosity determination.

Steady shear viscosity of the suspension of the polymer-hemoglobin was measured with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40). Shear rates were from 45 to 450 s^{-1} at 22 °C.

BOVINE HB PLA NANOCAPSULES
OXYGEN DISSOCIATION
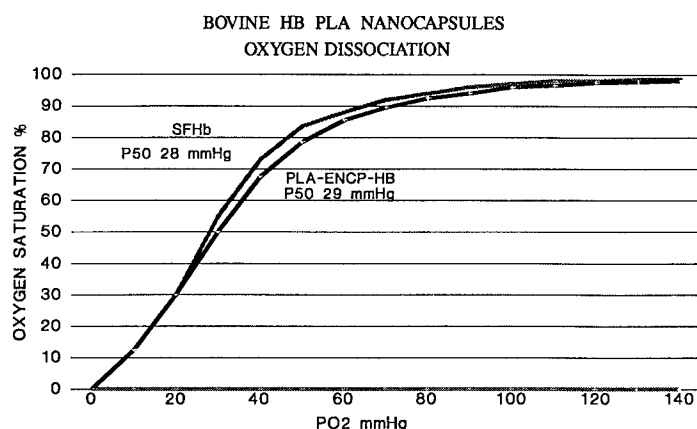
Figure 2. Oxygen dissociation curve for bovine hemoglobin and polylactide bovine hemoglobin nanocapsules.

Determination of oxygen affinity,size distribution & membrane thickness

The oxygen affinity curve was determined by the TCS Hemoxanalyser (TCS Medical Products Co., U.S.A.) at 37 °C. Size distribution was by using Gaussian Analysis with a mean run time of 75 secconds,average count rate of 350 KHz and channel width of 36 USEC. Membrane thickness was determined by electronmicroscopy.

RESULTS AND DISCUSSION

Biodegradable polymer membrane hemoglobin nanocapsules.

When ethanol and acetone mixed solution containing phospholipid and polymer was injected into hemoglobin solution, ethanol and acetone dispersed rapidly into water. This way, biodegradable polymer-lipid membrane was formed. The average particle size of biodegradable polymer membrane hemoglobin nanocpasule can be varied over a wide range. We prepared the optimal range of between 0.08 to 0.12 micron (80 - 120 nm). They are spherical and homogeneous. The membrane thickness is 0.005-0.015 micron (5-15nm). With different formulation, the hemoglobin contents in the particles may be varied from 30 - 45 %. Phospholipid is not required in bovine hemoglobin. It is required for human hemoglobin in order to retain cofactors required for optimal P50.

Oxygen affinity of biodegradable polymer membrane containing hemoglobin.

Oxygen dissociation curves were made using the TCS Hemoxanalyser. The P50 of the biodegradable polymer membrane containing bovine hemoglobin was between 27 - 29 mmHg. There was no significant difference in the oxygen dissociation of bovine hemoglobin solution and biodegradable hemoglobin nanocapsules. The P50 of the Hb-nanocapsules and the P50 of the bovine hemoglobin are in good agreement (Figure 2). This means that the procedure of preparation did not damage hemoglobin.

TABLE I. CHARACTERISTICS OF POLYMER-HEMOGLOBIN

Specific Gravity (22 °C)	1.0043
Viscosity (37 °C)	3.2 - 3.4 cp
P50(mmHg)	27 - 29
Hill's coefficient	2.4 - 2.9
Bohr coefficient	-0.22 to -0.24

The effects of the pH on oxygen affinity of hemoglobin nanocapsules were also analyzed. The Bohr effect was about -0.22 to -0.24. Other characteristics of the bovine hemoglobin nanocapsules is summarzied in Table I.

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TWO TYPES OF TOTALLY ARTIFICIAL RED BLOOD CELL SUBSTITUTES
LIPOSOME EMBEDDED HEME(L/H) AND
LIPIDHEME/MICROSPHERE(LHM)

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ABSTRACT

Two types of totally artificial oxygen carriers were produced
(1)by embedding synthetic lipidhemes (as oxygen carriers) in bi-
layers of liposomes as vehicles of lipidhemes(L/H) and
(2)by covering clinically available fat droplets (triglyceride
microspheres) with synthetic lipidhemes(LHM). Fat droplets were
used as vehicles of lipidhemes. Their oxygen carrying ability in
vivo was examined in beagles undergoing hemorrhagic shock.
L/H delivered 15.7 to 19.2 % of total oxygen delivery. From 12.7
to 24.4% of total oxygen consumption was from L/H. LHM delivered
11.6 to 7.3 % of total oxygen delivery. From 13.1 to 16.4 % of
total oxygen consumption was from LHM.
These totally synthetic red blood cell substitutes can be candi-
ates for futere clinical testing.

INTRODUCTION

 Development of red blood cell substitute is mandatory because
of shortage of blood and possible viral infections. Many red
blood cell substitutes have been reported. However, none of them
is clinically available so far. Most of red blood cell substitu-
tes compose of human or bovine hemoglobin as a functional part
to combine oxygen. Tsuchida's group produced synthetic lipid-
hemes as oxygen carriers. By molecularly assembling these lipid-
hemes they produced two types of totally synthetic red blood
cell substitutes. They were liposome embedded heme(L/H) and
lipidheme microsphere(LHM).
L/H system : As lipidhemes were very small they were embedded in
bilayers of liposomes 49± 13 nm in diameter(FIG.1)[1] . In this
system liposomes worked as vehicles of lipidhemes.
LHM system : Clinically available fat emulusion known to be safe
to human body was used as vehicles of lipidhemes. Lipidhemes
were arranged to cover around the surface of triglyceride fat
droplets 103± 29 nm in diameter(FIG.2.)[2] . The purpose of this
study was to evaluate oxygen carrying ability of these two total
ly synthetic red blood cell substitutes in dogs undergoing
hemorrhagic shock.

MATERIALS AND METHODS

 Beagles weighing about 8 kg were studied. They were anesthetiz-
ed, intubated and mechanically ventilated with a Harvad Resrira-
tor with a tidal volume of 25 ml/kg at a rate of 12/min. A Swan-
Ganz catheter was inserted via the internal jugular vein for
cardiac output measurements, mixed venous blood gas analysis and
measurements of hemoglobin(Hb) and L/H or LHM concentration in
the blood. A line was inserted via femoral artery for arterial
blood gas analysis. Then, 30 ml/kg of blood was shed via femoral
artery. After 30 minutes, in L/H Group(n=6) 30 ml/kg of L/H
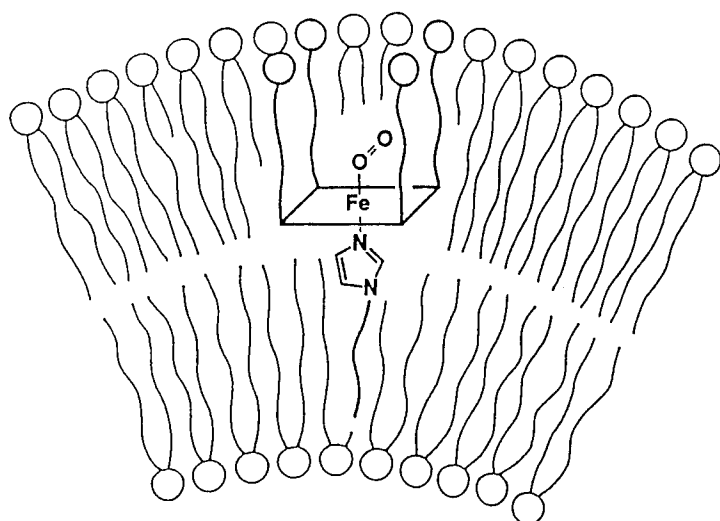
Liposome/heme (L/H)

FIGURE 1. Structure of lipidhemes embedded in bylayers of liposome(L/H).
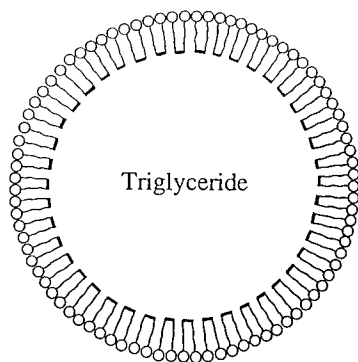
Lipidheme-Microsphere (LH-M)

FIGURE 2. Structure of lipidheme microspheres(LH-M).

Table I . OXYGEN DELIVERY AND OXYGEN CONSUMPTION IN L/H GROUP

	C O (1/min.)	DO_2(ml/min.) H b	L／H	%DO_2 %	VO_2(ml/min.) H b	L／H	%VO_2 %
0	0.8	120	—	—	57	—	—
IAI	1.5	180	43	19.2	50	16	24.4
30'	1.5	182	42	18.7	53	16	23.6
60'	1.5	185	39	17.3	53	14	20.8
90'	1.5	181	35	16.2	54	13	18.5
120'	1.4	171	32	15.7	53	10	15.8
180'	1.2	135	26	16.0	47	7	12.9

After administration of L/H, L/H delivered 26 to 43 ml/min. of oxygen corresponding to 15.7 to 19.2 % of total oxygen delivery. Oxygen consumption from L/H was 7 to 16 ml/min. which was 12.9 to 24.4 % of total oxygen consumption.

solution was injected intravenously. In LHM Group, 30 ml/kg of L/H solution was injected intravenously. Measurements were performed at 30 minutes after hemorrhage as control values of this study(0 time), immediately after injection(IAI) of L/H or LHM solution(IAI), 30, 60, 90, 120 and 180 minutes after injection of L/H or LHM solution. In L/H Grouop oxygen delivery(DO_2) by dogs' Hb and L/H, and %DO_2(oxygen delivered by L/H was divided total oxygen delivery) were calculated. Oxygen consumption(VO_2) from Hb and L/H, and %VO_2(oxygen consumed from L/H was divided by total oxygen consumption) were calculated. In the same way calculations were made in LHM Group.

RESULTS

Table 1 shows oxygen delivary by dogs' Hb and L/H, %DO_2, oxygen consumption from dogs' Hb and L/H and %VO_2.

Table Ⅱ. OXYGEN DELIVERY AND OXYGEN CONSUMPTION IN LHM GROUP .

C O (1/min.)		DO_2(ml/min.) Hb	L H - M	%DO_2 %	VO_2(ml/min.) H b	L H - M	%VO_2 %
0	0.8	127	—	—	51	—	—
IAI	1.0	146	26	15.1	52	10	16.1
30'	1.0	143	30	17.3	56	11	16.4
60'	1.0	148	24	13.9	53	9	14.5
90'	1.1	156	28	15.2	65	10	14.5
120'	1.0	141	24	14.5	55	9	14.1
180'	1.3	167	22	11.6	43	7	13.1

LHM delivered 22 to 30 ml/min. of oxygen corresponding to 11.6 to 17.3 % of total oxygen delivery. Oxygen consumption from LHM was 7 to 11 ml/min. which was 13.1 to 16.4 % of total oxygen consumption.

Table 2. shows oxygen delivery by dogs' Hb, LHM, and %DO_2, and oxygen consumption from dogs' Hb, LHM and %VO_2.

DISCUSSION

If red blood cell substitutes is produced, which does not need crssmatching, is free from diseases or virus and is easily stored and transported, it will be very beneficial for mankind. Both L/H and LHM have such characteristics. Komatsu reported L/H and LHM have specific gravity, viscosity and osmotic pressure very similar to that of human red blood cell. In vitro study, their oxygen binding was reversible and fast, their P_{50} was 40 mmHg, their solution properties were satisfactory to physiological needs[2]. Our in vivo study in beagles undergoing hemorrahgic shock demonstrated that both L/H and LHM had ability to load oxygen in the lung and unload it in the tissue. Thus, L/H and LHM can be candidates for future clinical testing.

KOBAYASHI ET AL.

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OXYGEN TRANSPORT AND *IN VIVO* PARAMETERS OF ARTIFICIAL RED CELLS (ARC)

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ABSTRACT

Artificial red cells (ARC) are prepared by encapsulating Hb with a polymerizable phospholipid. Their physical stability is very high and long-term preservation is possible in the frozen state. We examined the effect of blood parameters on the hematological and biochemical findings in transfused rats. The oxygen transport capacity of ARC *in vivo* were also tested by exchange transfusion in beagles. The oxygen binding parameters were almost the same as those of red blood cells (*i.e.*, P_{50}, Hill's coefficient, and oxygen transport efficiency (OTE) were 30 mmHg, 2.5, and 30%, respectively). The blood parameters after transfusion showed no significant changes when compared with the control. The oxygen transport capacity was of the same efficiency as red blood cells.

INTRODUCTION

Liposome encapsulated hemoglobins have been the focus of attention because of their potential for use as oxygen carriers using human hemoglobin. The features of low viscosity, low oncotic pressure, long half-life in the blood stream, controlled oxygen affinity, and high content of hemoglobin are attractive. However, some disadvantages exist (*e.g.*, lack of physical stability, leakage of hemoglobin, aggregation, and fusion) [1]. We stabilized the liposome membrane by polymerizing the unsaturated phospholipids that make up the membrane. The

hemoglobin encapsulated in the polylipid vesicles (*i.e.*, ARC) had excellent physical stability, and long-term preservation was possible in either the frozen or dried powder forms [2]. We have already reported on the results from studies on the blood compatibility of ARC [3-5].

The present study was done with *in vivo* systems to evaluate the oxygen transport capability of ARC and to obtain biochemical findings in the blood stream. The oxygen transport capacity was confirmed by exchange transfusion in beagles. The results indicated that the oxygen transport capacity of ARC is the same as the that of red blood cells and that ARC produce no adverse effects on the kidney and liver.

MATERIALS AND METHODS

Preparation of ARC

Stroma-free hemoglobin (SF-Hb) was prepared from human red blood cells. The final concentration and pH were adjusted to 30 g/dl and 7.4, respectively, with HEPES buffer. The SF-Hb was combined with carbon monoxide to prevent formation of met-hemoglobin during preparation. The ARC were prepared using a lipid mixture consisting of 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (DODPC), cholesterol, and 2,4-octadecadienoic acid (molar ratio of 7:7:2) along with SF-HbCO containing NADH as a reductant and allosteric effector. The solution was extruded through polycarbonate filters (pore size up to 0.2 μm) to encapsulate the SF-Hb with the lipid vesicles and then the untrapped Hb was removed by the dialysis method. The purified vesicles were irradiated by γ-radiation (5 kGy) at 4 °C to polymerize the lipid membranes. The hemoglobin concentration in the total suspension was adjusted to 7 g/dl. The entire procedure was carried out asceptically. The characteristics of the ARC, such as particle size, oncotic pressure, met-Hb content, viscosity, P_{50}, Hill coefficient, and the OTE which is defined as the difference between the oxygen saturation at PO_2 of 110mmHg and 40mmHg, were measured.

Measurement of blood parameters

An *in vivo* test of the blood parameters was performed on 6-week-old male rats (Crj;CD(SD)). Five rats were used for each of the control and treatment groups. An ARC solution was infused intravenously into the tails at doses of 4,000 mg/Kg body weight at an infusion rate of 1 ml/min. The control rats were only injected with a physiological saline of the same volume as the ARC solution infused into the treatment group. For hematological and biochemical examination, 2 ml of blood was drawn from the abdominal vein on 1, 3, 14, 30, 60, 90, and 105 days after treatment. The following hematological and

biochemical examinations were carried out: hematocrit, red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), white cell differential, concentration of hemoglobin (HGB) and ARC, concentration of total protein (TP), albumin (ALB), total bilirubin (BIL), total cholesterol (T-CHO), triglyceride (TG), phospholipid (PL), glucose (GLU), creatinine (CRE), albumin-globulin ratio (A/G), glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), alkaline phosphatase (ALP), lactic dehydrogenase (LDH), blood urea nitrogen (BUN), and electrolyte (Na^+, K^+, Cl^-). The resulting data were treated statistically.

Exchange transfusion

Beagles weighing 7.5-8.5 kg, (total blood volume: 600-700 ml) were anesthetized. A catheter was inserted into the femoral artery and then a Swan-Ganz catheter was inserted into the pulmonary artery through the jugular vein. Two-hundred milliliters of blood was drawn from the femoral artery and the same volume of ARC was infused into the pulmonary artery 20 min later. This treatment was repeated until the exchange ratio had reached 40% or 70%. After the transfusion, 10 ml of blood was periodically drawn from the femoral artery for arterial blood and from the pulmonary vein for venous blood to check the pH, PO_2, PCO_2, and ARC contents. The blood pressure, heart rate, and cardiac output (Q) were monitored during the entire procedure. The Hb contents in the red blood cells and ARC were determined by using the hematocrit and ARC-crit methods [5]. The oxygen saturations in arterial and venous blood (SaO_2 and SvO_2) of ARC were obtained for different oxygen partial pressures in arterial and venous blood (PaO_2 and PvO_2) from the ARC oxygen dissociation curve. The SaO_2 and SvO_2 of the red blood cells were determined in a similar manner this time from the human red blood cells oxygen dissociation curve. The oxygen consumptions (VO_2) of the red blood cells and ARC were calculated separately according to the following formula.

$$VO_2 \text{ (ml/min)} = 10 \times Q \text{ (l/min)} \times 1.39 \times [Hb] \times (SaO_2 - SvO_2) \qquad (1)$$

RESULTS

Characteristics of ARC

The characteristics of ARC are listed in Table I. The oxygen binding parameters was the same as those of human blood cells.

Measurement of blood parameters

Figure 1 shows the hematological findings. An increase in the leukocyte count and a decrease in the platelet count were observed up to 3 days after treatment. After 2 weeks, both of these parameters had recovered to the control

AKAMA ET AL.

TABLE I. Characteristics of ARC.

Particle size (μm)	0.22	P_{50} (mmHg)	30
Oncotic pressure (mOsm)	313	Hill coefficient (n value)	2.57
Viscosity (cp)	4.2	OTE (%)	30
Met-Hb content (%)	<1	Endotoxin (EU / ml)	<0.01
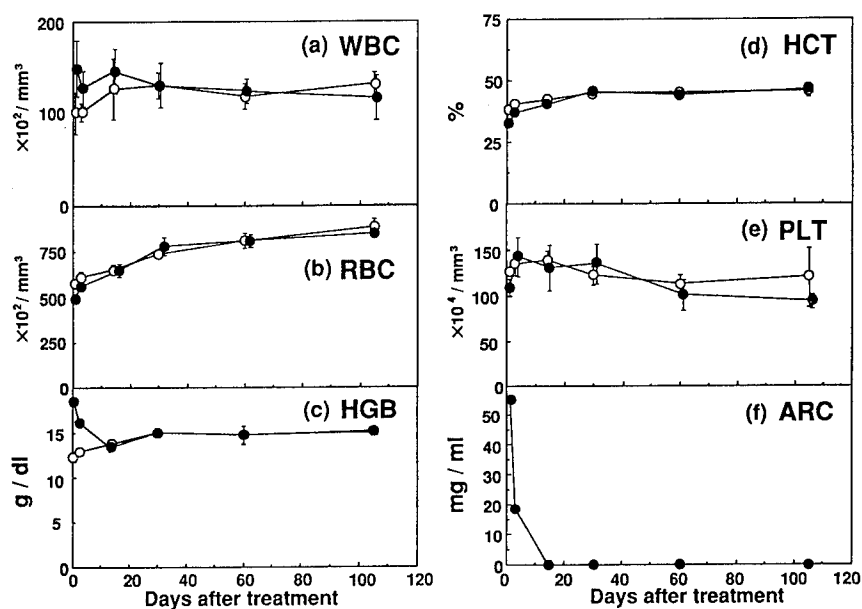
FIGURE 1. Hematological findings in rats after treatment with ARC.
(O) control, (●) ARC.

level. There were no significant changes in hematological parameters. Figure
1(f) shows the changes in the ARC contents in the blood after infusion. The
concentration of ARC were 55 mg/ml at day one after infusion. After 14 days, no
ARC were detected in the blood. The changes in the leukocyte count were slight
and only temporary. Figure 2 shows the biochemical findings. Increases in ALP ,
T-CHO and PL and decrease in TG were observed up to 30 days after treatment.
No obvious changes were seen in the values of GOT and GPT. The other blood
parameters after treatment with ARC were the same as control level.
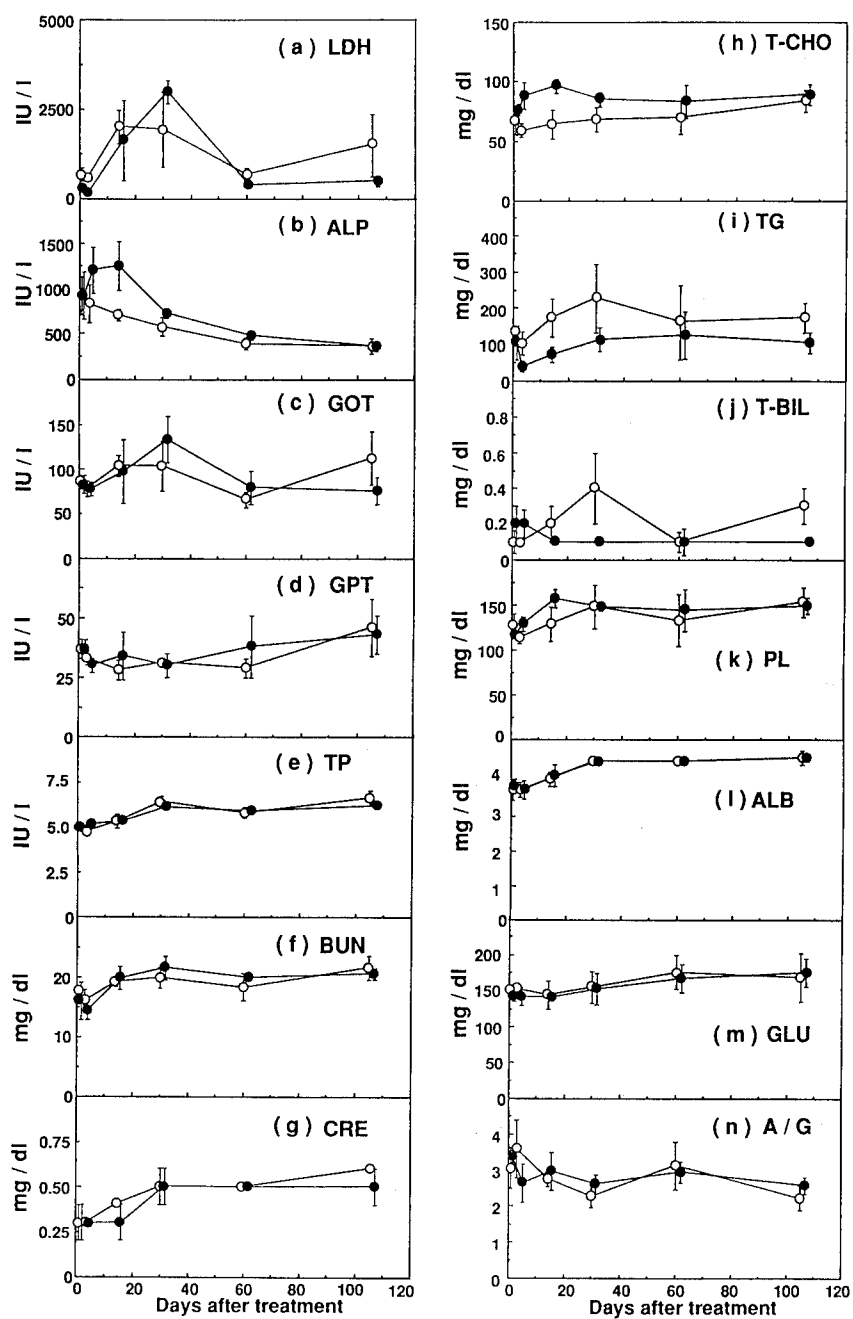
FIGURE 2. Biochemical findings in rats treatment with ARC. (○) control, (●) ARC.

TABLE II. Results of Hb content, cardiac output (Q), oxygen partial pressure (PO2), oxygen saturation (SO2), and oxygen consumption (VO2) before and after 70% exchange transfusion in the beagle. (**I**, before hemorrhage; **II**, 33% hemorrhage; **III**, 30 min after hemorrhage; **IV**, 33% exchange transfusion; **V**, 55% exchange transfusion; Time(h), after 70% exchange transfusion.)

							Time (h)					
		I	II	III	IV	V	0	0.5	1.0	1.5	2.0	3.0
Hb content (g/dl):	(Native red blood cells)	12.5	13.9	12.1	9.5	6.0	4.5	4.2	4.4	5.0	4.6	4.3
	(ARC)	—	—	—	2.8	3.8	4.4	4.4	4.2	4.4	4.4	4.2
Cardiac output (Q) (l/min)		1.61	0.53	0.60	0.91	1.24	1.29	1.40	1.15	1.20	1.18	1.22
PO2 (mmHg):	(arterial blood)	112.8	116.5	114.7	122.7	122.6	123.2	126.8	127.1	121.6	125.7	120.4
	(venous blood)	47.2	29.6	29.7	38.8	38.0	35.0	35.9	33.3	29.4	35.6	26.6
SaO2 (%)	(Native red blood cells)	98.1	98.2	98.0	98.0	97.9	97.4	97.9	98.1	97.9	98.1	98.0
	(ARC)	—	—	—	98.2	98.2	98.2	98.3	98.2	98.2	98.2	98.2
SvO2 (%)	(Native red blood cells)	81.8	50.1	47.8	64.0	61.5	50.1	54.8	51.9	43.1	55.3	38.7
	(ARC)	—	—	—	65.5	64.6	60.2	56.6	44.3	60.6	49.6	56.6
VO2 (ml/min):	(Native red blood cells)	45.6	49.1	50.8	40.8	37.5	37.7	34.8	32.2	36.4	29.5	31.6
	(ARC)	—	—	—	11.4	21.8	29.8	24.6	27.8	31.1	24.4	25.7

Exchange transfusion

Table II summarizes the results of cardiac output (Q), oxygen partial pressure (PO2), oxygen saturation (SO2), and Hb content and oxygen consumption (VO2) of the red blood cells and ARC in each blood sample for the 70% exchange transfusion in beagle. The cardiac output decreased to 0.5 at 33% of hemorrhage and recovered to the initial level after 70% transfusion. The oxygen consumption (VO2) for the red blood cells and ARC calculated according to Formula (1), which show that ARC can transport oxygen with the same efficiency as the red blood cells. The half-life of ARC in the blood stream at 40% and 70% exchange transfusion were obtained at 21 h and >50 h (approximate), respectively (data not shown). The beagles transfused to 40% were still alive after more than a year and showed no side effects.

DISCUSSION

Hematological parameters revealed only temporary changes. Biochemical findings relating to lipid metabolisms showed increases in ALP, T-CHO and PL and decrease in TG. There were no changes observed, however, in the GOT and GPT for kidney function and the BUN and CRE for liver function. The resulting blood parameters after transfusion in rats also revealed that there was no significant influence on the function of the kidney and liver. The results of exchange transfusions in beagles indicated that the oxygen transport capacity of

ARC was similar to that of red blood cells. In addition, the half-life values of ARC are adequate for blood substitutes.

In conclusion, the results obtained to date suggest that ARC have no significant influence on the blood parameters and a sufficient oxygen transport capacity that is similar to red blood cells. These attractive features should allow them to play an important role in transfusion in the future.

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CIRCULATION PROFILE OF TECHNETIUM-99m LABELED LIPOSOME ENCAPSULATED HEMOGLOBIN IN A 10% OR 50% RAT HYPOVOLEMIC SHOCK MODEL

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ABSTRACT

The circulation kinetics and organ biodistribution of liposome encapsulated hemoglobin (LEH) was determined non-invasively in hypovolemic rats using a technetium-99m (Tc-99m) liposome labeling procedure. Rats with an indwelling catheter had either 10% or 50% of their blood volume removed and replaced with an equal volume of LEH labeled with Tc-99m (Tc-99m LEH). Mean arterial pressures and heart rates were monitored throughout the experiment. Gamma camera images were acquired during the first 90 minutes following infusion of the Tc-99m LEH and at 20 hours post-infusion. Blood capillaries were drawn at various times to monitor Tc-99m activity and the amount of LEH remaining in circulation. There were significant differences in the kinetics and biodistribution for the two groups. In the 10% group, the LEH was removed quickly from circulation with only 0.9% remaining at 20 hours, while the 50% group had 51.2% at 20 hours. The liver and spleen were the major organs responsible for LEH clearance. The 20 hour biodistribution by tissue sampling showed that the 10% group had 37.6% in the liver and 34.3% in the spleen, while the 50% group had only 18.4% in the liver and 14.6% in the spleen.

INTRODUCTION

Liposome encapsulated hemoglobin (LEH) is under development as an oxygen-carrying resuscitative fluid for the delivery of oxygen to tissues under conditions of

blood loss following trauma, surgery, and combat casualty [1-3]. A technetium-99m (Tc-99m) labeling protocol for liposomes has been developed to aid in this progress by allowing the non-invasive determination of the circulation kinetics and organ biodistribution of LEH [4,5]. This labeling technique is advantageous over other liposome labeling methods because it is simple to perform, has a high labeling efficiency, has excellent *in vivo* stability, and can be performed on preformed liposomes. Initially, LEH biodistribution studies in rabbits were performed by infusing a 25% Tc-99m LEH dose on top of the normal blood volume and monitoring the circulation profile [4]. This earlier study was used to test the safety of the LEH and to determine LEH pharmacokinetics and organ biodistribution. In the present study, we have used this same liposome labeling technique to determine the circulation profile of Tc-99m LEH in a rat hypovolemic shock model, which better represents a physiological situation where LEH would be administered.

MATERIALS AND METHODS

LEH containing native bovine hemoglobin and glutathione was prepared and characterized as previously described [3-5]. The lipid composition of the LEH was distearoyl phosphatidylcholine, cholesterol, dimyristoyl phosphatidylglycerol, and α-tocopherol (50:40:9:1 mole ratio). Glutathione was added to the preparation to prevent the oxidation of hemoglobin to methemoglobin and for the Tc-99m liposome labeling. The liposomal diameter was 687 ± 31 nm by photon correlation spectroscopy. Hemoglobin concentration in the LEH was 0.46 mM and the methemoglobin level was 11 ± 4%. The phospholipid concentration was 100 mM. The LEH had an endotoxin level of 6<x<60 Eu/ml and was sterile.

LEH was labeled with Tc-99m using the lipophilic chelator, hexamethylpropyleneamine oxime (Ceretec™). The incubation mixture was washed in PBS and centrifuged at 30,000 x g to remove any free Tc-99m. Labeling efficiency was determined by dividing the activity associated with the LEH by the total activity initially added. The mean labeling efficiency after the initial wash for the Tc-99m LEH used in this study was 91.6 ± 0.8 %.

Male Sprague-Dawley rats (350-400 g) were anesthetized with Metofane for placement of an indwelling catheter in the femoral artery 2-3 days before the hypovolemic exchange experiments. The catheter consisted of 23 gauge Tygon tubing with a 28 gauge Teflon tip to aid in ease of placement. On the day of the exchange, the animals were weighed to determine their blood volume using 56

ml/kg body weight. The rats were anesthetized with 30 mg/kg of pentobarbital intraperitoneally and placed in a prone position on a Searle Model 6413 gamma camera equipped with a low-energy all-purpose collimator. Baseline mean arterial pressure (MAP) and heart rate measurements were obtained using a Grass Model 7D physiograph. Blood was withdrawn through the catheter at a rate of 1 ml/min. Two mls of blood were removed from the 10% rats, while 10-11 mls were removed in the 50% group. The additional time for removal of blood for the 50% group was added to the 10% group before the reinfusion of Tc-99m LEH through the tail vein at a rate of 1 ml/min. The 10% group received 2 mls of Tc-99m LEH (~1.5 mCi, 170 mg/ kg hemoglobin, 430 mg/kg phospholipid), while the 50% group received 10 mls (~3 mCi, 850 mg/kg hemoglobin, 2.2 g/kg phospholipid). One minute dynamic images were acquired over a 90 minute period from the beginning of the reinfusion. Heart rate and MAP were monitored continuously during the imaging period. Blood samples were drawn in capillary tubes at the end of the infusion as well as at 30, 60, and 90 minutes. At 20 hours, a static image was acquired; heart rate and MAP determined; a blood sample drawn; and then the rats were sacrificed. Tissue samples were removed, washed in saline, weighed, and counted in a scintillation well counter. A small sample of labeled LEH was used as a standard reference.

Images were analyzed with region of interest quantitation and corrected for isotope decay using software developed for the Pinnacle workstation. The image data of each organ was then corrected for the amount of activity contributed by the blood pool activity.

RESULTS

There were differences recorded in the MAP and heart rate for the two groups replaced with Tc-99m LEH (n=5 per group). The MAP was only slightly affected by the removal of 10% of the blood volume, which represents the donation of a unit of blood in humans. The MAP for the 10% group dropped from 105 ± 8 mmHg to 72 ± 10 mmHg following the bleeding period, recovered to 117 ± 6 mmHg at 90 minutes post-infusion, and dropped slightly to 92 ± 5 mmHg at 20 hours post-infusion. On the contrary, the MAP for the 50% group dropped from 97 ± 5 mmHg to 31 ± 5 mmHg during the bleeding period, recovered to 91 ± 7 mmHg at 90 minutes post-infusion, but dropped to 30 ± 14 mmHg by 20 hours. The MAP of rats (n=3) receiving 50% normal saline (0.9%) was measured and compared to the 50% LEH group to determine if LEH served only as a volume replacement.

The MAP for the saline rats dropped from 89 ± 5 mmHg to 31 ± 1 mmHg after the bleeding period, and recovered to 71 ± 6 mmHg at 90 minutes post-infusion. Unlike the 50% LEH group, the MAP for the saline rats continued to recover to 84 ± 4 mmHg by 20 hours. The heart rate of the 10% group dropped from 306 ± 6 beats/min to 294 ± 11 beats/min following the bleeding period. By 20 hours post-infusion, the heart rate of the 10% group was 371 ± 1 beats/min. In contrast to the 10% group, there was a greater drop in the heart rate for the 50% group at the end of the bleeding period from a baseline value of 324 ± 11 beats/min to 228 ± 20 beats/min. At 90 minutes post-infusion, the heart rate of the 50% group recovered to 336 ± 11 beats/min and dropped slightly to 312 ± 34 beats/min by 20 hours. There was a similar drop in heart rate for the saline group as for the 50% LEH group following the bleeding period (320 ± 8 beats/min to 210 ± 13 beats/min). At 90 minutes post-infusion, the heart rate of the saline group recovered to 280 ± 20 beats/min and continued to increase to 380 ± 20 beats/min by 20 hours.

Figure 1 depicts serial scintigrams for two rats receiving either a 10% or 50% volume replacement with Tc-99m LEH following an equivalent amount of blood loss. There was a significant difference in the clearance kinetics for the two groups (n=5 per group). In the 10% rat, the Tc-99m LEH was cleared rapidly from the heart, which represents the circulating blood pool, with only 54.5% of the activity remaining by 90 minutes and 0.9% by 20 hours. In contrast, the 50% rat had a substantial amount of Tc-99m activity remaining in the heart with 87.9% of the Tc-99m LEH remaining in circulation at 90 minutes and 51.2% remaining at 20 hours.

There were also differences in the organ biodistribution patterns for the two groups. As shown in figure 1, the reticuloendothelial system (RES) organs of the liver and spleen were the major organs responsible for LEH clearance for both groups. Although the activity in the liver and spleen in the 50% rat images appears to be equal to that of the 10% rat, correction for the blood pool contribution showed that the accumulation of Tc-99m LEH in the liver was only 7.2% and 17.1% at 90 minutes and 20 hours, respectively. Spleen accumulation for the 50% group was 8.3% and 17.1% at 90 minutes and 20 hours, respectively. On the contrary, the 10% rat had less Tc-99m LEH remaining in circulation which could contribute to the blood pool activity of the liver and spleen. There was 25.4% and 37.5% in the spleen for the 10% group at 90 minutes and 20 hours, respectively. Liver accumulation for the 10% group was 17.0% and 36.2% at 90 minutes and 20 hours, respectively.
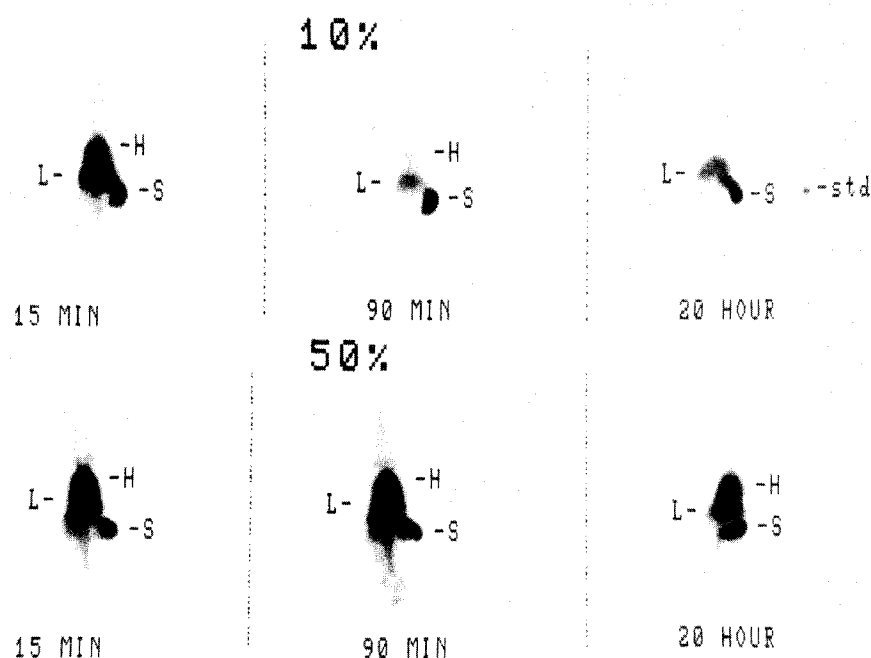
FIGURE 1. Gamma camera images depicting the circulation profiles at various times for rats following replacement of either 10% or 50% of their blood volume with Tc-99m LEH. Heart (h), Liver (l), Spleen (s).

There was a 100% survival rate for the two groups during the experimental period. Figure 2 shows the biodistribution obtained from autopsy of the animals at 20 hours. The overall organ biodistribution pattern determined by tissue sampling agreed with the image biodistribution results. Of significance were the large differences in activity in the blood, liver, and spleen for the two groups.

DISCUSSION

In this study, we have monitored the clearance kinetics and organ biodistribution of Tc-99m LEH in rats during two experimental stages of hypovolemia. The LEH clearance rate of the 10% group in this resuscitation model was significantly faster than that of the 50% group. This clearance rate difference directly leads to the
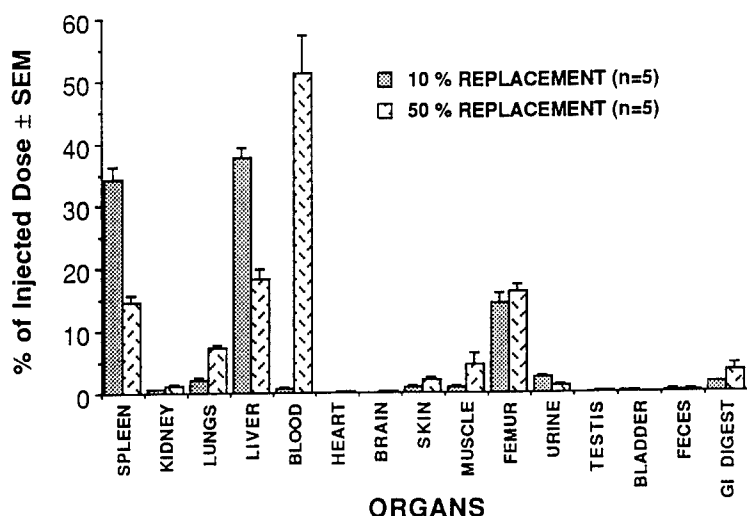
FIGURE 2. Autopsy organ biodistribution at 20 hours for rats (n=5 per group) following replacement of 10% or 50% of their blood volume with Tc-99m LEH.

different organ biodistribution for the two groups. Although the liver and spleen were the major organs responsible for the removal of Tc-99m LEH from circulation for both groups, the removal by these organs was much slower for the 50% group. One explanation for the different rates is the dose-dependent clearance of the Tc-99m LEH by the RES. Another reason for the observed differences may be that a blood volume feedback mechanism in the 50% group may actually decrease the rate of removal of the Tc-99m LEH by the RES organs. Thirdly, the difference may be due to a physiologic decrease in the function of the RES secondary to the physiologic stress of a large exchange volume. Further studies will be performed to address the mechanism of Tc-99m LEH clearance in hypovolemia.

ACKNOWLEDGEMENTS

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III. LATE PAPERS

DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb™):
BIOANALYTICAL STUDIES IN SWINE.

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ABSTRACT

These studies were a part of preclinical safety and efficacy studies of DCLHb. Their purpose was to analyze the characteristics of DCLHb during circulation, and the distribution of iron following the administration of DCLHb to swine. Swine were dosed (2 g/kg) with 10 g/dL DCLHb, infused intravenously at a rate of 1 mL/kg/min. Blood samples were collected up to 48 hours post-infusion for analysis. Tissue samples were obtained for iron determination. The data collected showed that the concentration of DCLHb in the plasma gradually decreased, while the concentration of methemoglobin remained low and essentially constant. The oxygen binding characteristics and stability of the crosslink were preserved following infusion, indicating that the DCLHb continued to function as an effective oxygen carrier. Iron concentrations in the liver and kidneys increased as expected, but plasma levels of iron did not saturate the iron binding capacity of transferrin, inferring a controlled process for the release of iron.

INTRODUCTION

DCLHb is a potential blood substitute currently in clinical trials. There are three chemical and physical characteristics of infused DCLHb impacting its effectiveness as an oxygen carrier: rate of oxidation, the *in vivo* oxygen binding

917

characteristics (P_{50}) and the stability of the crosslink (affecting elimination and P_{50}). Apart from DCLHb's oxygen transport capabilities, the distribution of iron at intervals post-infusion was investigated to determine if the release of iron was a controlled process.

MATERIALS AND METHODS

Twelve Yorkshire/Landrace crossbred pigs were dosed (2g/kg) with DCLHb intravenously at a rate of 1 mL/kg/min. Blood samples from eight pigs (sacrificed at 48 hours post-infusion) were collected pre- and post-infusion, and at 2, 4, 8, 24, and 48 hours post-infusion, and centrifuged to obtain samples of plasma. The plasma hemoglobin concentration was determined and the hemoglobin characterized. The oxygen binding characteristics of plasma hemoglobin in a post-infusion sample were compared to those of a DCLHb test article. To profile tissue iron accumulation with time, tissue samples were obtained from two pigs sacrificed at 24 hours post-infusion and from two pigs sacrificed at 96 hours post-infusion and compared with the tissues from two swine which were not dosed. Plasma for serum iron determinations was obtained from swine subsequently sacrificed 96 hours post-infusion at the following intervals: pre- and post-infusion, and at 2, 4, 8, 24, 32, and 96 hours post-infusion.

Spectrophotometry was used to determine total plasma hemoglobin, oxyhemoglobin, and methemoglobin. The UV-visible spectra of samples diluted in phosphate buffered saline were obtained. Literature values for the extinction coefficients at characteristic absorptions of hemoglobin [O.W. van Assendelft and W.G. Zijlstra, Anlyt Biochem 69: 43-48, (1975)] were used to calculate the concentrations of each of these hemoglobins.

Size-exclusion chromatography (SEC) was used to resolve DCLHb from native hemoglobin and other proteins using the following conditions: Column, Superose® 12 HR 10/30; Mobile Phase, 0.75 M MgCl2 / 50 mM BIS TRIS / 0.1 mM EDTA / pH 6.5; Flow rate, 0.1 mL/min; Detection, 414 nm.

Oxygen equilibrium curves were obtained using a Hemox Analyzer (TCS Medical Products) operated at 37°C. The infused DCLHb in plasma sample was diluted in pre-injection plasma from the same pig and Bis Tris buffer was added to maintain the DCLHb/plasma near pH 7.4. Control DCLHb solutions were diluted in TCS Hemox buffer (135 mM NaCl, 5 mM KCl, 30 mM TES).

To determine serum iron, plasma samples were treated with a mixed acid reagent (HCl, TCA, and thioglycollic acid) to dissociate iron from transferrin, reduce Fe(III) to Fe(II), and precipitate proteins. To correct for the small amount of hemoglobin iron susceptible to this treatment, results were corrected by an amount indicated from the spiking of plasma samples with known amounts of hemoglobin. The iron in the supernatant was complexed with ferene and absorbances measured at 592 nm. The average total iron binding capacity (TIBC) of the plasma samples was determined. Iron (in slight excess as $FeCl_3$) was added to saturate transferrin with iron. Then magnesium carbonate was added to precipitate excess iron. The resulting supernatant was treated with the mixed acid reagent and analyzed as above. Tissues were digested in hot nitric acid and the total iron concentration determined by ICP-Atomic Emission Spectroscopy.

RESULTS

The concentrations in the plasma of DCLHb and the oxidized fraction were plotted (Figure 1). Immediately post-infusion the concentration of plasma DCLHb was 2.3 g/dL. The concentration gradually decreased (to 1.2 g/dL after 8 hours, 0.7 g/dL after 24 hours and 0.3 g/dL after 48 hours). The concentration of plasma methemoglobin remained low at approximately 0.1 g/dL, peaking slightly at a concentration of 0.2 g/dL one day post-infusion. The oxyhemoglobin curve shows that a very significant portion of the DCLHb in circulation at any time post-infusion was capable of carrying oxygen.

From the plasma oxygen binding curve obtained immediately post-infusion, the oxygen binding properties were characterized by an oxygen affinity (P_{50}) of 36 mmHg and cooperativity (n) of 2.4. Both the oxygen affinity and
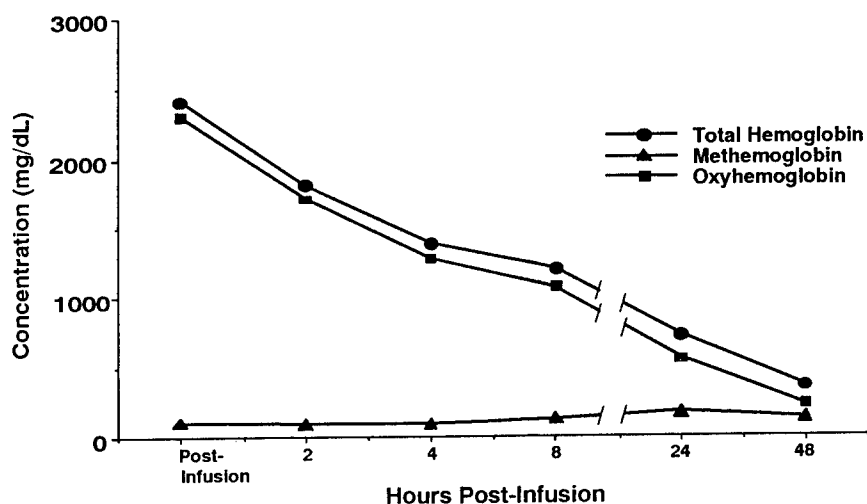
FIGURE 1   PLASMA HEMOGLOBIN CONCENTRATION CURVES

cooperativity describe plasma oxygen binding properties closely matching that of the DCLHb test article.

In the mobile phase employed for the size exclusion chromatographic separations, uncrosslinked hemoglobin dissociates completely into $\alpha\beta$ dimers (MW 32,250) while DCLHb, because of its covalent crosslink, retains its tetrameric configuration (MW 64,500). The chromatographic system is capable of resolving the $\alpha\beta$ dimers from the stabilized tetramer. The hemoglobin in the plasma of DCLHb-infused animals was identified as crosslinked on the basis of chromatographic retention.

The integrated area of the DCLHb peak correlated during the post-infusion period with the plasma hemoglobin concentration obtained spectrophotometrically. (This method could also be used to detect random occurrences of hemolysis during sample preparation.) There was no indication of crosslink instability, i.e., dimer formation, up to 48 hours post-infusion.
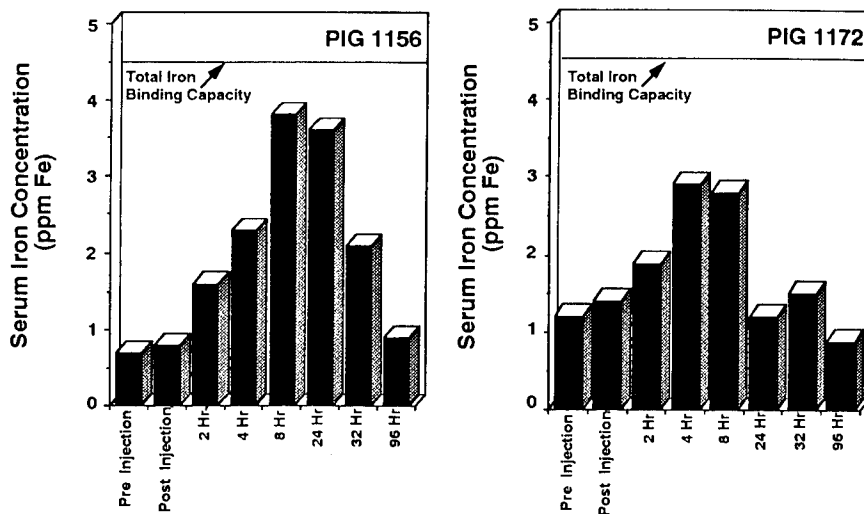
FIGURE 2   SERUM IRON PROFILES

Serum iron profiles were obtained for two individual animals (Figure 2). The serum iron concentrations increased to a maximum at approximately 8 hours post-infusion and then decreased to baseline values by 96 hours post-infusion. At no time interval measured did the serum iron concentrations exceed the total iron binding capacity determined as approximately 4 ppm iron.

The tissue iron profiles show a different trend (Figure 3). Iron continued to accumulate in both the kidney and liver between 24 and 96 hours post-infusion. The concentration of iron in DCLHb-dosed animals at 96 hours was approximately double that of undosed pigs.

DISCUSSION

Taken together, the lack of rapid oxidation coupled with the maintenance of oxygen binding characteristics *in vivo* indicated that DCLHb continued to function as an effective oxygen carrier after infusion. The *in vivo* stability of the
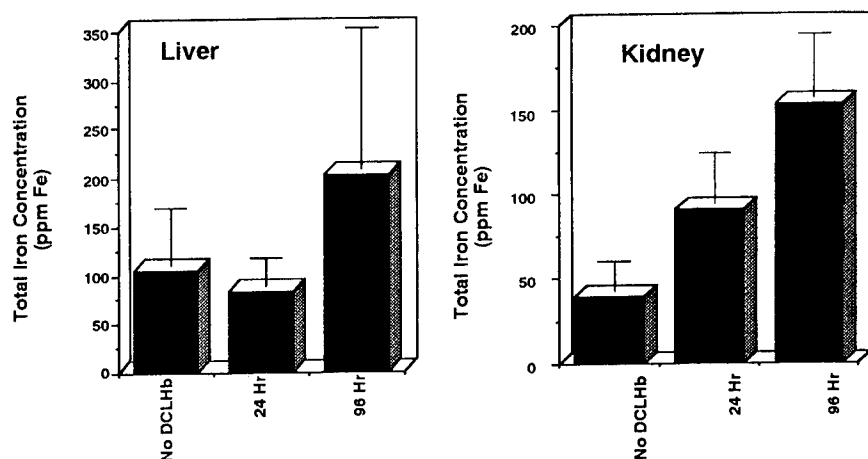
FIGURE 3   TISSUE IRON PROFILES

crosslink during circulation was anticipated, given the chemical, mechanical and thermal stability demonstrated by the crosslink during *in vitro* testing.

The gradual increase and subsequent decline in serum iron concentrations was such that the TIBC of the plasma was not exceeded. Transferrin actively transports iron in the period of 2 to 32 hours after infusion and the release of iron appeared to be controlled. Iron was accumulated in the liver and kidneys.

DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb™) POLYMERIZATION

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ABSTRACT

By employing proprietary polymerization agents possessing specific binding groups and by completing diaspirin crosslinked hemoglobin (DCLHb) polymerization under specific conditions, we have selectively achieved the following objectives: (1) the P_{50} was adjusted to the physiologic range or left- or right-shifted; (2) the surface of DCLHb was modified ("decorated"); (3) DCLHb was polymerized but not decorated; (4) DCLHb was polymerized and decorated; or (5) DCLHb was site-specifically modified and polymerized.

INTRODUCTION

Polymerization of hemoglobin offers the benefits of reduced renal clearance and metabolism and increased duration in the vascular circulation. However, the scientific literature also points out two major deterrents to hemoglobin polymerization: (a) inefficiency of polymerization; and (b) increase oxygen affinity of the resulting polyHb. We have addressed these concerns by employing proprietary polymerization agents possessing specific binding groups and by completing diaspirin crosslinked hemoglobin (DCLHb) polymerization under specific conditions.

EXPERIMENTAL DESIGN

Three polyethylene glycol-based (PEG-based) polymerization agents were used to demonstrate that polymerized DCLHb (polyDCLHb) could be prepared to have different properties such as P_{50} value, surface decoration, and polymerization. The first of these polymerization agents, DENACOL™ (Nagase Chemical Co.), reacts randomly with DCLHb, whereas the other two agents, BMAA-PEG and ITP-PEG-PTI, react at specific sites on DCLHb.

ANALYTICAL METHODS

Size exclusion chromatography (SEC) and reversed-phase HPLC (RP-HPLC) were used to monitor the polymerization of DCLHb and analyze the polyDCLHb products. SEC was performed using TSK-G4000SW and TSK-G3000SW columns connected in series and a mobile phase consisting of a 9:1 (v/v) ratio of 50 mM phosphate buffer, pH 6.5, and 2-propanol, delivered at 1 mL/min. Analytes were monitored at 280 nm. Alternatively, SEC was performed using a Superose™-12 column (Pharmacia), a mobile phase consisting of 50 mM phosphate buffer, pH 6.5, delivered at 0.2 mL/min with analyte detection at 280 nm. RP-HPLC was carried using a Vydac Protein-C_4 column (4.6 mm ID and 250 mm length) and a combined mobile phase delivered at a flow rate of 1 mL/min as a linear gradient of B in A, where A was CH_3CN / H_2O / TFA (20:80:0.1, by volume) and B was CH_3CN / H_2O / TFA (60:40:0.1, by volume). The gradient consisted of the following steps: (a) 50% B to 55% B over 20 minutes; (b) 55% B to 75% B over 10 minutes; and (c) 75% B to 85% B over 10 minutes. Analyte detection at 220 nm was employed.

Thiol groups were determined by the method of Neis *et al.* (Toxicology 31, 1984; 319).

Each polyDCLHb sample was diluted in Hemox solution (30 mM TES buffer, 130 mM NaCl and 5 mM KCl), and the oxygen equilibrium curve was obtained using a Hemox Analyzer Model B operated at a cell temperature of 37°C. The oxygen affinity value (P_{50}) was determined directly from the

oxygen-binding curve, and the cooperativity value (n_{40-60}) was calculated from the Hill plot.

DENACOL-POLYMERIZED DCLHb

Synthesis. The pH of 19 g/dL DCLHb in Ringer's lactate solution was adjusted to pH 9.0 by the addition of 1 M sodium carbonate. The solution was deoxygenated by successive vacuum / nitrogen cycles for 1 hour at 25°C. Denacol was added and the reaction mixture was stirred under nitrogen at 25°C or 5°C. The reaction was monitored by SEC. At 5°C, the molar ratio of Denacol:DCLHb ranged from 40:1 to 60:1 and the reaction was quenched with N-acetyl-L-cysteine after 4 or 5 days; whereas at 25°C the molar ratio ranged from 15:1 to 25:1 and the reaction time was a day or less. The polymer solution was dialyzed against Ringer's lactate solution to give final product. Experimental results are summarized in Figure 1a and Table I.

Results and Discussion. In-process SEC analysis of the reaction with Denacol indicated that the retention time of DCLHb was shifted toward the higher molecular weight region during reaction; thus, DCLHb was decorated by Denacol. The SEC profile of the final product (Figure 1a) shows that the product contains polymerized and decorated DCLHb. Polymerization of deoxyDCLHb with Denacol at 5°C yielded a polyDCLHb having a left-shifted oxygen dissociation curve relative to DCLHb (or fresh blood). P_{50} values were in the range of 30-40 mm Hg, depending on the reaction parameters. However, both polyDCLHbs that were obtained from similar reactions completed at 25°C had right-shifted oxygen dissociation curves, with P_{50} values in the range of 60-80 mm Hg (Table 1).

If the goal is completely polymerized DCLHb, Denacol is not an efficient polymerization agent. Even under optimal conditions, using 15-60 equivalents of Denacol, polyDCLHb products still contained 40-50% of surface-modified ("decorated") and not polymerized DCLHb. However, the remaining 50-60% of the product was decorated polyDCLHb.

1a. PolyDCLHb from Denacol

Retention Time (Minutes)

1b. PolyDCLHb from BMAA-PEG

Retention Time (Minutes)

1c. PolyDCLHb from ITP-PEG-TPI

Retention Time (Minutes)
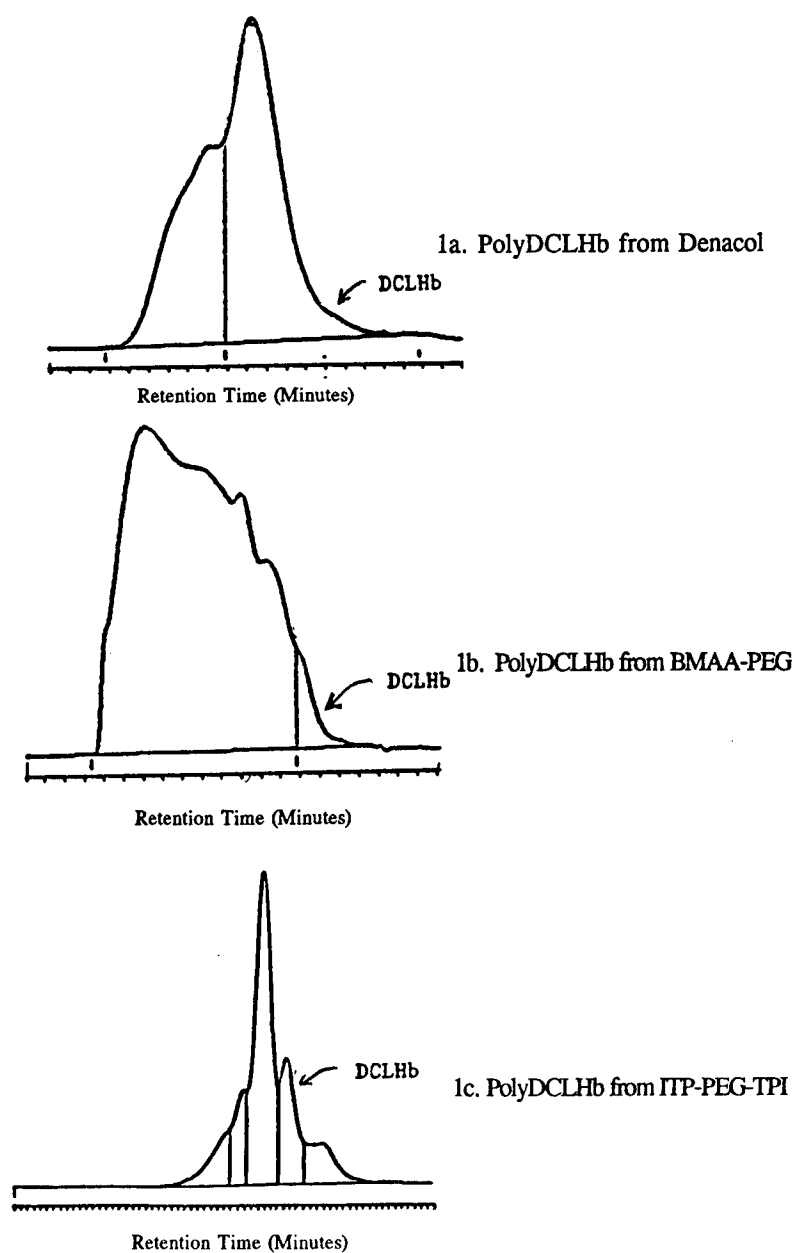
FIGURE 1   SEC Analysis of PolyDCLHb

TABLE I. Characteristics of Denacol-Polymerized DCLHb (DP-DCLHb)

Molar Ratio Denacol/DCLHb	Reaction Temperature (°C)	Reaction Time (Hr)	SEC (TSK) Profile (%)		P_{50} (n)
			Decorated DCLHb	Decorated Oligomers of DCLHb	
15:1	25	24	51	49	62 (1.3)
25:1	25	24	46	54	50 (1.3)
40:1	5	120	45	55	40 (1.2)
60:1	5	120	41	57	31 (1.2)

BMAA-PEG-POLYMERIZED DCLHb

Synthesis. A solution of 20 g/dL DCLHb in pH 7.0 buffer was deoxygenated by vacuum / nitrogen cycles at 25°C. Polymerization was initiated by the addition of BMAA-PEG in deoxygenated water. The reaction was monitored by SEC. After 5 hours, polymerization was terminated by the addition of N-acetyl-L-cysteine at 5°C under nitrogen. The resulting solution was dialyzed against Ringer's lactate solution to give final product. Experimental data are summarized in Table II and Figures 1b and 2.

Results and Discussion. In contrast to observations during reactions with Denacol, in-process SEC analysis of the reaction of BMAA-PEG with DCLHb showed that the retention time of DCLHb did not shift toward the higher molecular weight region. Thus, BMAA-PEG does not decorate DCLHb. Three equivalents of BMAA-PEG gave polyDCLHb product containing only about 3% residual DCLHb (Figure 1b). This demonstrates that BMAA-PEG is an efficient polymerization agent. The P_{50}'s of polyDCLHb derived from reaction with BMAA-PEG were about 20 mm Hg. RP-HPLC analysis (Figure 2) suggests that BMAA-PEG binds specifically to the ß-chains of DCLHb at the cysteine-ß93 residues. A specific peak, which could be a modified ß-chain (ß'), was detected

TABLE II. Characteristics of BMAA-PEG-Polymerized DCLHb

Molar Ratio BMAA-PEG: DCLHb	% Composition by SEC (TSK)			RP-HPLC Profile			Titrable -SH	P_{50} (n)
	DCLHb	(DCLHb)$_2$	(DCLHb)$_{>2}$	ß'/Heme	ß/Heme	αα/Heme		
0	100				2.8 (100%)	3.5 (100%)	1.74	32 (2.6)
2	10	18	70	0.8	0.9 (30%)	3.2 (90%)	0.59	21 (1.8)
2.5	5	13	80	0.9	0.7 (25%)	3.1 (90%)	0.43	20 (1.7)
3	3	11	83	1.0	0.6 (20%)	3.1 (90%)	0.32	18 (1.7)

The components (DCLHb)$_2$ and (DCLHb)$_{>2}$ are dimers and oligomers of DCLHb, respectively. In the RP-HPLC data, ß' signifies modified ß-subunits, and the numbers in parentheses are the percent of unmodified ß- or αα-chains.

under all conditions, and its intensity increased with the molar ratio of BMAA-PEG to DCLHb. In contrast, αα-chains were practically unmodified. This hypothesis of specific ß-chain modification is confirmed by analysis of thiol groups: 50% of the reactive thiol groups were modified by two equivalents of BMAA-PEG.

ITP-PEG-PTI-POLYMERIZED DCLHb

Synthesis. DCLHb (10 g/dL) in Ringer's lactate solution was dialyzed against the desired buffer. Five (5) mL of the resulting solution having the selected pH was deoxygenated by successive vacuum / nitrogen cycles at room temperature. A solution of ITP-PEG-TPI in the same deoxygenated buffer was added, and the reaction mixture was stirred at room temperature under nitrogen. The course of reaction was monitored by SEC using a Superose-12™ column. After 24 hours the reaction mixture was cooled to 5°C. Simultaneous methemoglobin reduction and quenching were effected by the addition of deoxygenated 1 M N-acetyl-L-cysteine. The reaction mixture was stirred at 5°C overnight and dialyzed against Ringer's lactate solution to give final product. Experimental results are summarized in Table III and Figure 1c.
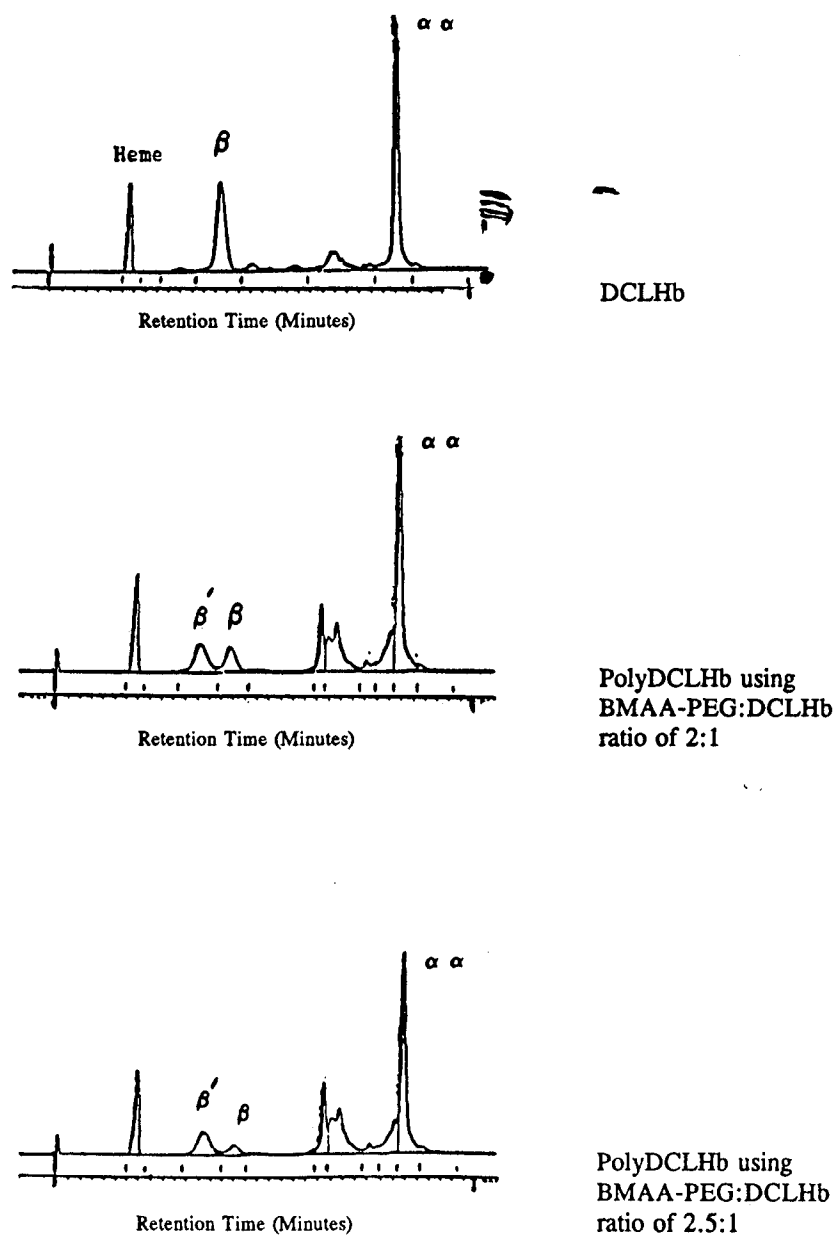
FIGURE 2 RP-HPLC Analysis of PolyDCLHb From BMAA-PEG

TABLE III. Characteristics of ITP-PEG-PTI-Polymerized DCLHb

Reaction Conditions		% Composition by SEC (Superose-12)			RP-HPLC Profile		P_{50} (n)
Buffer pH	Molar Ratio ITP-PEG-TPI: DCLHb	DCLHb	(DCLHb)$_2$	Other Oligomers	ß/Heme	$\alpha\alpha$/Heme	
	0	97	3		2.05 (100%)	1.38 (100%)	32 (2.6)
9.0	2.6	47	29	24	0.98 (48%)	1.20 (87%)	23 (1.7)
9.0	5.1	20	35	45	1.09 (53%)	1.20 (87%)	25 (1.9)
8.0	5	19	48	34	1.37 (67%)	1.20 (87%)	38 (2.0)
7.5	5	16	50	34	1.43 (69%)	1.30 (94%)	40 (2.1)
7.0	5	13	50	37	1.29 (63%)	1.20 (87%)	37 (1.7)
7.0	5	20	48	32	1.32 (64%)	1.20 (87%)	34 (1.7)

The components (DCLHb)$_2$ and (DCLHb)$_{>2}$ are dimers and oligomers of DCLHb, respectively. In the RP-HPLC data, ß' signifies modified ß-subunits, and the numbers in parentheses are the percent of unmodified ß- or $\alpha\alpha$-chains.

Results and Discussion. ITP-PEG-TPI gave polyDCLHb in which a dimer, (DCLHb)$_2$, was the major product (Figure 1c). This result is in agreement with theory, since ITP-PEG-TPI is a bifunctional affinity polymerization agent having high negative charge density at each terminus. The following observations strongly indicate that ITP-PEG-TPI covalently binds to DCLHb at the 2,3-DPG site. (a) The highest yield of the target product (DCLHb)$_2$ is obtained at a low buffer pH where interaction with cationic centers in the 2,3-DPG site is anticipated (Table III). (b) RP-HPLC analyses indicate that the ß-subunits are predominantly modified by this agent. The 2,3-DPG site is formed by portions of the ß-subunits. (c) The shifts in oxygen affinity of the oligomeric product parallel those anticipated by reaction at the 2,3-DPG site, i.e., binding at this site

would be expected to right-shift the oxygen binding curve and the lower the P_{50}, whereas less specific binding would have less predictable effects on the oxygen binding curve. (d) SDS-PAGE analyses of the product give bands corresponding in molecular weight to $\beta\beta$-, $\alpha\alpha$- and β-subunits, with decreases in the intensity of the β-subunit response compared to those of DCLHb.

LIPIDHEME-MICROSPHERE (LH-M)

A NEW TYPE OF TOTALLY SYNTHETIC OXYGEN CARRIER

AND ITS OXYGEN CARRYING ABILITY

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ABSTRACT

We have succeeded in synthesizing a new type of totally artificial oxygen carrier which was produced by covering oil droplets (microsphere) with synthetic hemes (LH-M).
We studied its oxygen-transporting ability in hemorrhagic dogs.

Four beagles weighing about 8 kg were studied. Under controlled ventilation, exchange-transfusion of 30 ml/kg was carried out. Cardiac output, hemoglobin and LH-M concentration in the blood, and blood gas were measured to 5 hours after intravenous injection of LH-M solution.

LH-M delivered 15.7 to 22.3 ml/min (11 to 16 %) of oxygen to the tissue and 5.5 to 8.2 ml/min (11 to 17 %) of oxygen was consumed from LH-M to 5 hours after intravenous injection. Its half-life time in the blood stream was about 12 hours.

It was confirmed that LH-M transported oxygen and released it to the tissue in vivo.

933

INTRODUCTION

We have recently succeeded in synthesizing a new type of totally artificial oxygen carrier. It was produced by covering oil droplets (microsphere), which is clinical use for fat emulsion, with synthetic hemes (lipidheme-microsphere : LH-M). Its particle size and heme concentration are able to be controlled voluntarily and its viscosity is much lower than that of human blood.

The aim of this study is to examine oxygen transporting ability of LH-M in vivo.

MATERIALS AND METHODS

Four beagles weighing about 8 kg were studied. They were anesthetized with 200 mg of ketamine hydrochloride administered intramuscularly and 3 ml of pentobarbital sodium administered intravenously.

A cuffed endotracheal tube was inserted, and the beagles were connected to a Harvard respirator and mechanically ventilated at 12 breaths/min with a tidal volume of 25 ml/kg under ambient air.

A Swan-Ganz catheter and an arterial line were inserted for measurements of cardiac output, blood gas, and other blood samplings.

From each dog, 30 ml/kg of blood was shed. After 30 minutes, 30 ml/kg of LH-M solution was intravenously injected. Cardiac output, blood gas were measured to 5 hours after intravenous injection. Hemoglobin and LH-M concentration were determined by the cyanomet-heme method.

DO_2 (oxygen delivery by hemoglobin and LH-M), $\%DO_2$ (DO_2 by LH-M was divided by total DO_2), VO_2 (oxygen consumption from hemoglobin and LH-M), and $\%VO_2$ (VO_2 from LH-M was divided by total VO_2) were calculated respectively.
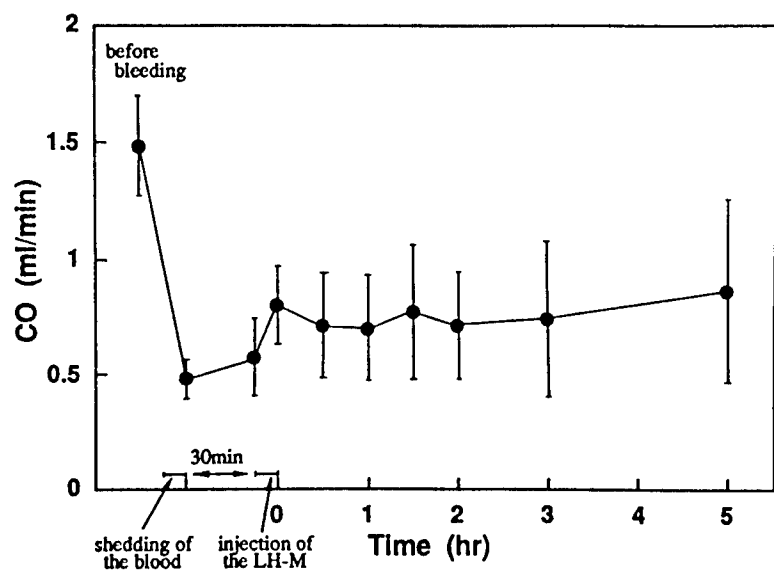
Cardiac output with time.
(n=4)

FIGURE 1   Cardiac output with time. (n=4)

RESULTS

Fig.1 shows changes of cardiac output (CO). The value of "before bleeding" was referred as control level. Thirty minutes after bleeding, the CO decreased to about 30 % of the control level. Immediately after the LH-M injection, the CO recovered to about 55 % of the control level, and 5 hours after injection, it was about 60 % of the control level. Throughout this experiment, no infusion was performed.

Fig.2 shows changes of oxygen delivery. LH-M delivered 15.7 to 22.3 ml/min of oxygen to the tissue ($\%DO_2$: 11 to 16 %) to 5 hours after intravenous injection.
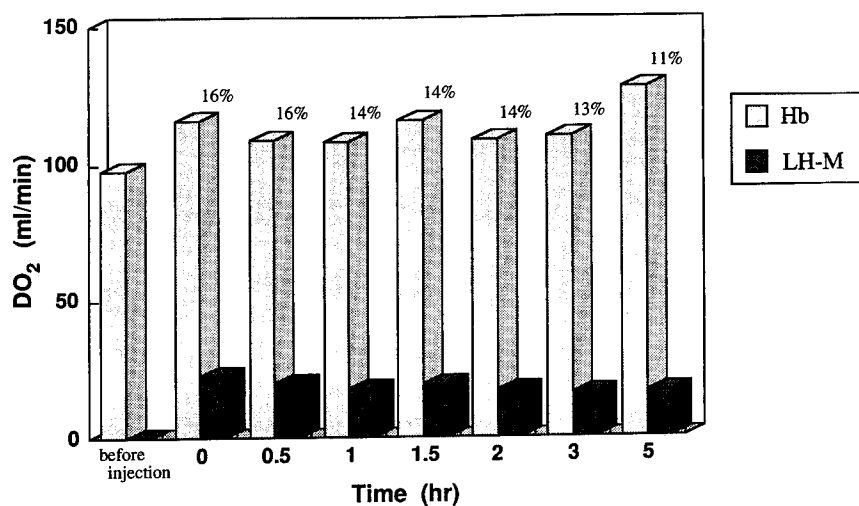
O_2 delivery by the hemoglobin and the LH-M after
injection of the LH-M solution.

(n=4)

FIGURE 2 O_2 delivery by the hemoglobin and the LH-M
after injection of the LH-M solution. (n=4)

Fig.3 shows changes of oxygen consumption. The oxygen volume consumed from LH-M was 5.5 to 8.2 ml/min ($\%VO_2$: 11 to 17 %) to 5 hours after intravenous injection.

Fig.4 shows half-life time of LH-M in blood stream, which was about 12 hours.

DISCUSSION

In vitro study, this lipidheme-microsphere (LH-M) has the characteristics as follows by Tsuchida et al.. (1) Totally synthetic system. (2) Oxygen binding is reversible and very
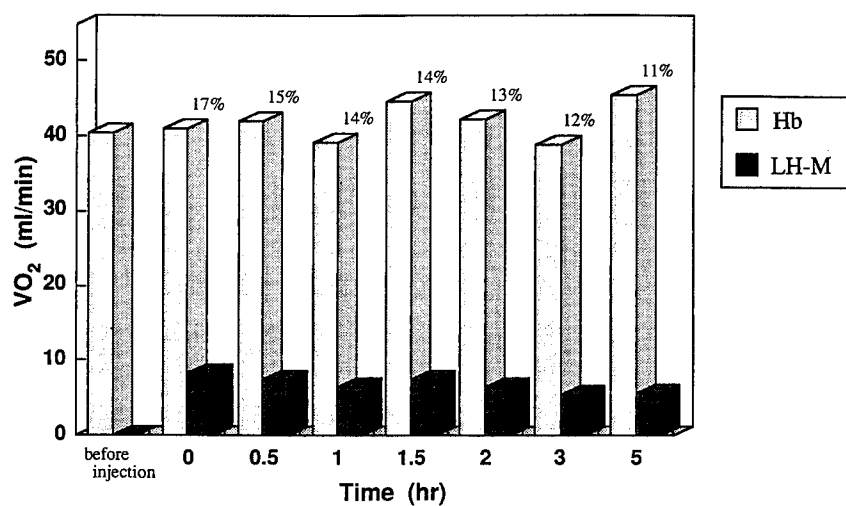
O$_2$ consumption$_{from}$the hemoglobin and the LH-M
after injection of the LH-M solution.

(n=4)

FIGURE 3   O$_2$ consumption from the hemoglbin and the LH-M

after injection of the LH-M solution. (n=4)
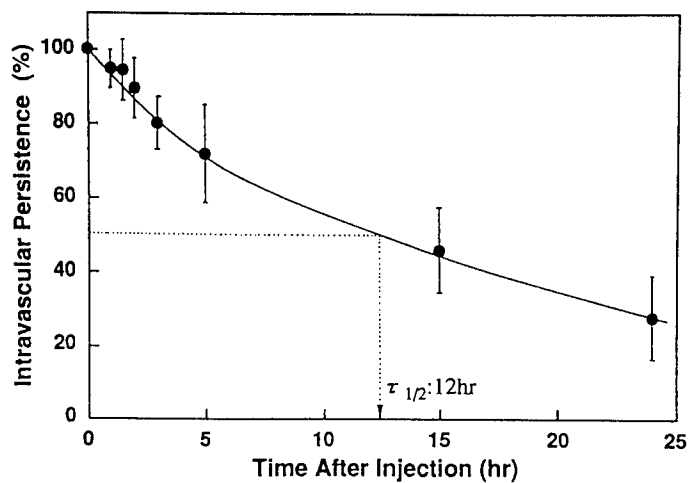
Half-life of the LH-M in blood stream.

FIGURE 4   Half-life of the LH-M in blood stram.

fast. (3) The oxygen volume dissolved in the fluid is similar or superior to that of blood. (4) The oxygen binding affinity is close to that of blood. (5) The solution properties are satisfactory to physiological need. (6) Long term storage stability.[1][2]

In our study, it was revealed that the LH-M had ability to transport oxygen to the tissue and released it to the tissue, even in vivo, and its half-life time in blood stream was about 12 hours. This type of totally artificial oxygen carrier such as the LH-M solution may be available for hemorrhagic shock state in clinical use.

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THE QUALITY CONTROL OF STROMA-FREE HEMOGLOBIN: LYSOPHOSPHATIDYLCHOLINE, A COMPONENT OF STROMAL PHOSPHOLIPIDS, AS CANDIDATE VASOCONSTRICTIVE FACTOR

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We characterized stromal phospholipids in stroma-free hemoglobin (SFH) by normal-phase and cation-exchange HPLCs, and found that SFH contained not only four phospholipids which were the major constituent classes in membrane, but also several peaks which were not yet identified. The residual amounts of these lipids in SFH were changed with storage of red cell concentrates. The four major phospholipids decreased concomitantly with storage, whereas the unidentified peaks increased after 21 days and then decreased after 48 days. We also found that SFH contained lysophosphatidylcholine (LPC) at 5.71 μg/ml, which was the deacylated metabolite of phosphatidylcholine (PC). These results suggest that stromal phospholipids are degradable.

Since LPC is known to be capable of producing a defect in endothelium-dependent arterial relaxation, we next examined the effect of stromal lipids on vascular tone in rabbit thoracic aortic strips. Preincubation with the crude lipid extract or the LPC purified from SFH by TLC significantly inhibited acetylcholine (ACh)-induced relaxation in phenylephrine (PhE)-precontracted tissues. These observations have led to the proposal that LPC, a component of stromal phospholipids, induces vasoconstriction as a result of inhibition of endothelium-dependent vasorelaxation.

INTRODUCTION

SFH prepared from outdated human red cells has been used as the starting materials for the hemoglobin (Hb)-based artificial oxygen carriers, because stromal components induce blood coagulation, renal failure, increase in blood pressure and numerous other problems (1). However, SFH or chemically modified red cell substitutes still cause unexpected reactions including vasoconstriction (2). The precise mechanisms involved in the observed toxicities remain to be elucidated. However, some of them might be due in part to impurities such as residual stromal phospholipids; indeed SFH still contains small quantities of phospholipids (3). The quantitation and characterization of stromal phospholipids are encouraged. In the present study, we characterized stromal lipids by HPLCs, and examined their roles in producing vasoconstriction in vitro.

MATERIALS AND METHODS

Preparation of Hb solutions- The preparation of SFH was described previously (3). Briefly, washed red cells obtained from outdated human red cell concentrates were lysed by the addition of two volumes of cold distilled water. After the hemolysate was left at 4°C for 10 min, the ionic strength was increased to the isotonic level by the addition of NaCl. This hemolysate was centrifuged at 36,000 x g for 30 min to obtain the 36,000 x g supernatant as a conventional SFH. A highly purified SFH was prepared by filtration of the hemolysate with a virus removal filter having a mean pore size of 35 or 40 nm (BMM-35, or BMM-40, Asahi Chemical Inc. Co., Ltd., Tokyo, Japan). To examine the effect of storage of red cell concentrates on residual stromal phospholipid in SFH, SFH was prepared from red cell concentrates which had been stored for 1, 21, and 48 days. In this experiment, red cell concentrates were stored without any additive solutions. Therefore, 21 days old is out-of-date.

Analysis of Phospholipids- Total stromal lipid was extracted according to the method of Bligh and Dyer (4) with a minor modification (3). The final extract was reconstituted in chloroform–methanol (1:1, v/v), and stored at -80°C until use. Radioactive [^{14}C]-phosphatidylinositol (Amersham, Buckinghamshire, England) was added as a tracer prior to lipid extraction to assess the extraction efficiency.

The four major constituent phospholipids, phosphatidylserine (PS), phosphatidylethanolamine (PE), PC, and sphingomyelin (SM) were separated using normal-phase HPLC. HPLC was performed with an analytical column of TSKgel Silica-60 (250 mm x 4.6 mm I.D., Toyo Soda, Tokyo, Japan). Lipid extract was separated isocratically using acetonitrile-methanol-phosphoric acid-water (900:95:2.75:1) at 30°C at a flow-rate of 1.1 ml/min, and the absorption at 210 nm was monitored.

LPC was analyzed by cation-exchange HPLC (5). Lipid extract was separated onto a Whatman Partisil 10 SCX column (250 mm x 4.6 mm I.D., Clifton, NJ, U.S.A.) with a guard column packed with a similar SCX pellicular media. Phospholipids were eluted with a mobile phase comprised of acetonitrile-methanol-water (400:100:40) at 30°C at a flow-rate of 2.5 ml/min.

Standard phospholipids were purchased from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Avanti Polar Lipids (Birmingham, AL, U.S.A.).

In vitro Examination of Stromal Lipids Using Rabbit Aorta- Rabbits of either sex (Japanese White; 2.0-3.0 kg) were anaesthetized with pentobarbitone (60 mg/kg) and sacrificed by exsanguination. Thoracic aortic strips of 3 mm in width were mounted in an organ bath under 1 g of tension in Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, CaCl$_2$ 1.8, MgCl$_2$ 2.1, NaH$_2$PO$_4$ 0.4, NaHCO$_3$ 11.9, glucose 5.6 (pH 7.4). This solution was aerated with air and all experiments were carried out at 37°C. The tissues were precontracted with phenylephrine (PhE, 1 µM), and acetylcholine (ACh, 1 µM) was added to elicit endothelium-dependent relaxation. To examine the effects of lipids, the tissues were preincubated for 1 hr in the presence of lipids, and then the responce to ACh was recorded in the same manner. Lipids used were egg yolk LPC (Sigma), the crude extract of SFH lipids, and the LPC purified from SFH lipids by TLC.

RESULTS

Analysis of Stromal Phospholipids by HPLCs- The detection limit of four major phospholipids on normal-phase HPLC was 0.01 for PS and PE, 0.03 for PC, and 0.2 µg for SM in the injected volume, respectively. The separations of stromal phospholipids in Hb solutions prepared from outdated red cell concentrates were shown in Fig. 1. The content of phospholipids in SFH (BMM method) was 0.32 ± 0.04 for PS, 0.42 ± 0.13 for PE, 1.93 ± 0.17 for PC, and 0.48 ± 0.07 µg/ml for SM (means ± SE of 6 experiments, Hb 8.1 g/dl), respectively. Several unidentified peaks (Ul
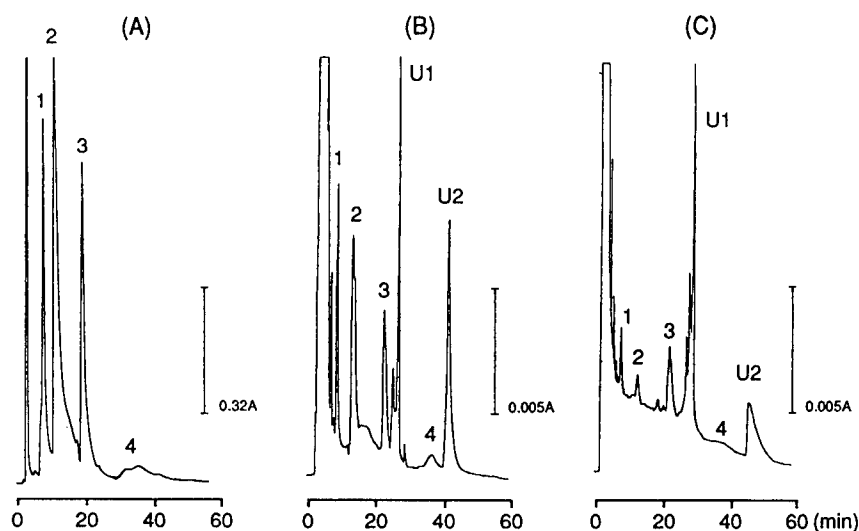
Fig.1 Normal-phase HPLC of lipids in (A) hemolysate, (B) the 36,000 x g supernatant, and (C) SFH prepared by the filtration with BMM. 1: phosphatidylserine; 2: phosphatidylethanolamine; 3: phoaphatidylcholine; 4: sphingomyerin; U1 and U2: unidentified peaks.

and U2 in Fig. 1) were also observed in the conventional and highly purified SFHs, whereas these peaks were relatively minor in the hemolysate.

The determination of lysophospholipids by normal-phase HPLC was interfered with by major phospholipids and the above mentioned unidentified peaks. The determination of LPC was achieved by cation-exchange HPLC (Fig. 2). The detection limit was 5 μg. Even by this HPLC method, the retention times of LPC and SM were relatively close. Therefore, the determination of LPC in the hemolysate and the 36,000 x g supernatant was impossible, since the presence of a relatively great amount of SM interfered with the determination of LPC. LPC sometimes showed two peaks on HPLC as shown in Fig. 2(A). The same phenomenon was also observed, when some of LPC standards and the LPC purified from SFH were separated on HPLC. This may be due to the heterogeneity in the composition of fatty acid.

Table 1 shows the changes in the content of phospholipids and unidentified peaks in the conventional SFH on storage at 4°C. The four major constituent phospholipids were decreased with storage, whereas the unidentified peaks were increased after 21 days and then decreased after 48 days. In a preliminary study, some of these unidentified peaks were elevated after heating (60°C for 2 hr) or acidification (pH 3.5 for 2 hr) of SFH. One of these peaks was separated by TLC and determined by the secondary ion-mass spectrometric method to be diacylglycerides having a mean molecular weight of 531.4. Further detailed characterization of these peaks is currently under investigation. In another experiment, we determined LPC content in SFH (BMM method) prepared from fresh or outdated red cell concentrates to be 5.00 ± 0.29 or 4.00 ± 0.81 μg/ml (mean ± SE of three measurements), respectively. There was no significant difference.
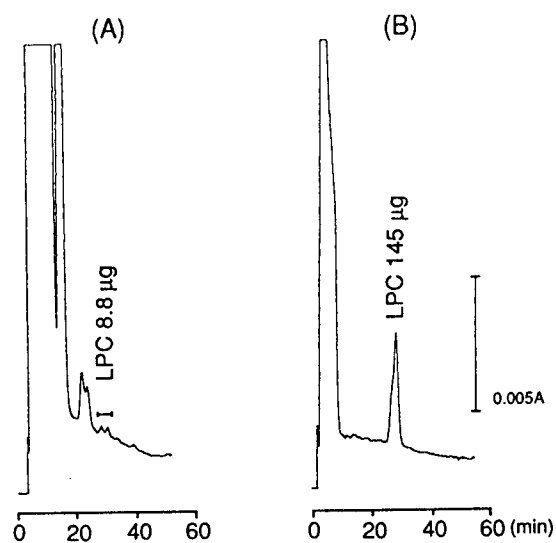
Fig.2 Cation-exchange HPLC of (A) stromal lipids and (B) lysophosphatidylcholine purified from SFH by TLC.

Table I. Contents of four major phospholipids and unidentified peaks in SFH on storage

lipids	1 day	21 days	48 days
phospholipids			
PS	2.62±1.40	1.24±0.74	0.26±0.06
PE	6.93±0.97	3.08±1.31	0.68±0.28
PC	7.43±0.65	5.23±1.71	1.74±1.36
SM	11.25±3.07	3.62±0.33	0.75±0.26
unidentified peaks			
U1	511.8±110.5	1456.8±214.6	371.2±120.2
U2	675.6±102.4	1540.3±394.7	236.4±108.9

Phospholipid content: μg/ml, unidentified peak content: peak integral/ml, mean±SD (n=4).
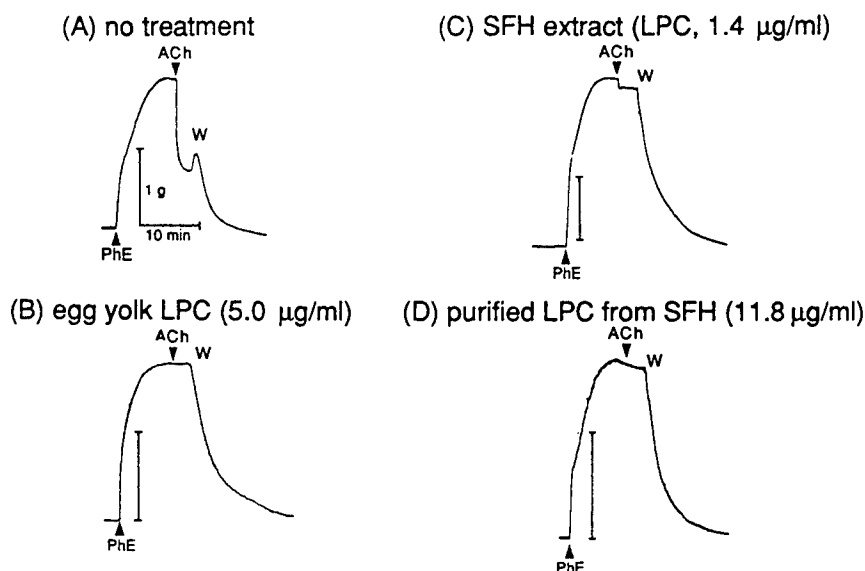
Fig.3 Inhibition of acethylcholine-induced relaxation by preincubation with lipids. (A) no treatment; (B) egg yolk lysophosphatidylcholine (LPC); (C) the crude lipid extract from SFH; (D) the LPC purified from SFH. PhE: phenylephrine 1 μM; ACh: acethylcholine 1 μM; W: washout.

Effect of Stromal Phospholipids on Vascular Tone- In the isolated rabbit aortic strips precontracted with PhE, ACh induced a relaxation dependent on endothelium. In contrast, preincubation of the tissues with egg yolk LPC, the crude extract of SFH lipids, or the LPC purified from SFH significantly inhibited the relaxant effect of ACh (Fig. 3). In a typical experiment, the LPC concentrations of lipid preparations were 5.0, 1.4, and 11.8 μg/ml, respectively. Virtually LPC at higher concentrations abolished ACh-induced relaxation. This ACh-induced relaxation was restored by the addition of sodium nitroprusside (0.01 mM, Sigma), an endothelium-independent vasodilator. The response to ACh was also reversed by the incubation for several hours in the absence of lipids.

On the other hand, four major phospholipids and two other lysophospholipids derived from PS and PE had no effect on endothelium-dependent relaxation evoked by ACh, when examined in a similar manner.

DISCUSSION

Two major findings have been obtained in the present study. First, the characterization of stromal phospholipids on normal-phase HPLC revealed that, in addition to the four major constituent phospholipids, several UV peaks were detected in the 36,000 x g supernatant and highly purified SFH. The four major phospholipids in the 36,000 x g supernatant decreased with storage of red cell concentrates. However, the unidentified peaks were increased after 21 days, and then decreased after 48 days. These results suggest that stromal phospholipids are degradable during storage of red cells. The unidentified peaks were supposed to be further degraded after 48 days.

The second finding is that SFH also contains LPC at 5.71 µg/ml, which is the deacylated metabolite derived from PC. Degradation of phospholipids in red cell membrane occurs during storage even in a frozen state. Ways (6) reported that the quantity of lipid phosphorus extractable from red cells decreased after more than two weeks at -20°C. However, this phenomenon didn't occur if Hb-free red cell ghosts were tested, suggesting the involvement of lipid peroxidation catalyzed possibly by Hb. He also found an accumulation of free fatty acid in the extracts, suggesting deacylation of phospholipids. Oxidatively modification of PC in low density lipoprotein also generates LPC (7). Therefore, we supposed lysophospholipids as the candidates for the above unidentified UV peaks observed on normal-phase HPLC. The examination by cation-exchange HPLC demonstrated the presence of LPC in SFH (Fig. 2). Whether other classes of lysophospholipids are contained in SFH is not yet determined. We had also tried to separate some of the unidentified peaks by TLC, and gotten some preliminary results by the secondary ion-mass spectrometric method. It showed a possibility that diacylglycerides were present in SFH. Although more quantitative examinations are necessary to decide whether diacylglycerides are major or minor component in SFH, the presence of LPC, and possibly diacylglycerides, may reinforce the possibility of degradation of stromal phospholipids.

LPC is already demonstrated to inhibit endothelium-dependent vasorelaxation (7,8). We confirmed that the crude extract of SFH lipids and the LPC purified from SFH inhibited ACh-induced relaxation in rabbit aortic strips. Currently, basal level EDRF formation is understood as a major factor which lowers systemic blood pressure in vivo by continuously relaxing resistance vessels. Inasmuch as LPC purified from SFH inhibited the EDRF-mediated vasorelaxation, LPC might be one of the vasoconstrictive factors present in SFH.

Previous studies have shown that small quantities of phospholipids and other stromal components derived from red cell membrane are still present in SFH (3,9,10); therefore, SFH is actually a stroma-reduced hemoglobin solution. We clarified that SFH contained several unidentified UV peaks and LPC, in addition to major constituent phospholipids. This shows the degradation of stromal phospholipids. Although the contributions of such degraded lipids to SFH-induced toxicities are not yet fully understood, LPC might be a candidate vasoconstrictor by inhibiting endothelium-dependent vasorelaxation. The significance of the quality control on stromal phospholipids is emphasized.

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